



## DOCTOR OF HEALTH (DHEALTH)

### **The potential usefulness of Hepcidin as a biomarker in patients presenting with iron deficiency anaemia (IDA)**

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*Award date:*  
2018

*Awarding institution:*  
University of Bath

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**UNIVERSITY OF  
BATH**

**The potential usefulness of Hepcidin as a biomarker in patients  
presenting with iron deficiency anaemia (IDA)**

Volume 1 of 1

Martin Gonzo

A thesis submitted for the degree of Doctor of Health

University of Bath

Department for Health

December 2017

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## **ACKNOWLEDGMENTS**

I would like to acknowledge the Institute of Biomedical Sciences for offering me a grant to purchase laboratory reagents used in this study, findings of which will in no doubt contribute to the existing knowledge base on Heparin. I would also wish to thank the Namibia Blood Transfusion Service (NaMBTS) and the Ministry of Health and Social Services (MoHSS) in Namibia for allowing me to carry out research on the Namibian population.

The efforts of my two supervisors Dr Gordon Taylor and Dr Aaron Maramba cannot go unnoticed. I am greatly indebted to the input my supervisors contributed in making this research a success. Their critical review of my numerous drafts was very constructive.

I wish to acknowledge Faithfull my wife and our dear children Tarnia, Tatenda and Tadiwanashe for enduring extended hours of my absence in the family. Thank you for encouraging me to soldier on even when I was exhausted. Your love and understanding has paid off.

## ABSTRACT

Anaemia is a global health concern affecting billions of people worldwide. In developing countries the predominance of nutritional iron deficiency significantly contributes to the problem of anaemia and this is indicated in the high prevalence of iron deficiency anaemia in the general population. Despite being a major problem, treatment of iron deficiency anaemia is not costly and can be administered with relative ease. The diagnosis of iron deficiency anaemia however, requires a multidisciplinary approach within the medical laboratory sections consequently resulting in the generation of a multitude of test results. The diagnostic approach to iron deficiency anaemia can be streamlined in an attempt to increase diagnostic sensitivity and to reduce uncertainties in interpreting laboratory generated data thus, the main aim of this study was to establish the usefulness of Hepcidin in the diagnosis and prognosis of iron deficiency anaemia. The other aim was to establish Hepcidin reference normal range values in Namibia for use in this study.

In establishing the reference normal range, a total of forty healthy adult participants were randomly selected from eligible blood donors. Forty-five participants with iron deficiency anaemia were also randomly selected to characterise the quantitative behaviour of Hepcidin in iron deplete states. More participants with iron deficiency were recruited compared to the number for healthy participants in order to cater for sample attrition. After accounting for sample attrition, a total of forty participants with iron deficiency who were on treatment were tested.

The introduction of Hepcidin testing in a routine diagnostic laboratory has potential in improving the diagnosis and prognosis of iron deficiency anaemia. In this study, serum Hepcidin reference normal range values for adults were established to be 17.803-86.181ng/mL among blood donors in Namibia. Participants with features suggestive of iron deficiency anaemia had suppressed serum Hepcidin values when compared to the established reference normal range. The serum Hepcidin concentrations however increased to normal levels on following up iron deficient participants who had been on iron treatment. The dynamics of Hepcidin was consistent with levels of serum iron in the same group indicating the potential

usefulness of Hepcidin in assessing iron bioavailability in iron deficiency anaemia. Correlation analysis indicated moderate but better associations between haemoglobin and Hepcidin compared to haemoglobin and serum iron levels. Anaemia had a better effect on serum Hepcidin ( $r=0.112$ ) than serum iron ( $r=0.015$ ) strengthening the case for using Hepcidin. Multiple regression analysis of the iron deficiency anaemia group before and after iron treatment showed statistically significant positive correlation between pre-treatment Hepcidin and serum iron ( $r= 0.456$   $p=0.003$ ). Similarly, a moderate positive association between  $\Delta$  Hepcidin and  $\Delta$  serum iron ( $r= 0.319$ ) was shown confirming the consistent association of baseline Hepcidin with serum iron.

## LIST OF ABBREVIATIONS

BMP:	Bone Morphogenetic Proteins
DMT:	Divalent Metal Transporters
EDTA:	Ethylenediamine Tetra Acetic Acid
ELISA:	Enzyme Linked Immunosorbent Assay
FBC:	Full Blood Count
HAMP:	Hepcidin Antimicrobial Peptide
HB:	Haemoglobin
HCT:	Haematocrit
Hepcidin:	hepcidin 25
HFE:	High Iron Fe
HJV:	Hemojuvelin
HRP:	Horse Radish Peroxidase
IDA:	Iron Deficiency Anaemia
MAP:	Mitogen Activated Protein
MCH:	Mean Corpuscular Haemoglobin
MCHC:	Mean Corpuscular Haemoglobin Concentration
MCV:	Mean Corpuscular Volume
MoHSS:	Ministry of Health and Social Services
NaMBTS:	Namibia Blood Transfusion Service
NUST:	Namibia University of Science and Technology
SMAD:	Sons of Mothers against Decapentaplegic
TFR:	Transferrin receptor
TMB:	Tetramethylbenzidine
WHO:	World Health Organisation

# CHAPTER 1

## 1. INTRODUCTION

Anaemia is characterized by a reduction in haemoglobin levels with or without accompanying reduction in red cell counts. The reduction could either be due to depressed rate of production or accelerated rate of destruction of the red blood cells. There are different types of anaemia and these are usually classified according to the aetiology of the anaemia. Iron deficiency can cause iron deficiency anaemia (IDA) because iron is required for the production of haeme which is the main component of haemoglobin. According to Hoffbrand and Moss (2011), Iron deficiency anaemia (IDA) is a haematological disorder characterised by a reduced level of haemoglobin which is attributed to inadequate iron bioavailability. In IDA, anaemia is due to depressed erythropoietic activity secondary to inadequate iron bioavailability. In the absence of sufficient amounts of iron, fewer red cells are produced which consequently result in reduced haemoglobin levels. Inadequate iron bioavailability can also cause production of morphologically distinct red cells which are microcytic, hypochromic and sometimes pencil shaped. These morphological features are a result of the significant reduction in haemoglobin content in the cells so produced.

Globally more than 2.2 billion individuals were affected with anaemia as of 2010 (Kassebaum et al., 2014). This accounts for more than 30% of the entire population worldwide of which iron deficiency anaemia was the most common cause (Kassebaum et al., 2014). In our published work on donor deferrals, anaemia was found to be the main reason with almost 50% of deferred donors attributed to low haemoglobin (Gonzo et al., 2016). This goes on to show the magnitude of the anaemia problem even in presumably healthy populations such as blood donors.

Towards the beginning of the twenty first century a report by UN and WHO indicated that in the adolescence populace there were about 2.5 cases of iron deficiency anaemia for every case of anaemia due to other causes (UNICEF/UNU/WHO 1998). According to WHO (2001) 50% of anaemia cases are due to nutritional iron deficiency.

More recent WHO prevalence studies also concur that more than 40% of anaemia in children is attributable to iron deficiency (WHO 2015). It also confirms that about 50% of anaemia in women could be eliminated by iron supplementation (WHO 2015). These figures suggest that anaemia is a health concern. Anaemia, regardless of aetiology, can negatively affect productivity and quality of life of the affected individuals due to reduced tissue oxygenation. Reduced tissue oxygenation occurs as a result of inadequate haemoglobin which consequently results in reduced cellular oxidative capacity. Iron deficiency anaemia in particular, results in decreased physical work capacity, athletic performances and cognitive impairment (Hlatswayo et al 2016).

Global prevalence data presented by Miller (2013) show that 64.6% of children in Africa are affected by anaemia. Iron deficiency anaemia (IDA), one of the anaemias is even more relevant in African developing countries and Namibia is not an exception. The WHO prevalence data on anaemia shows that anaemia due to nutritional deficiencies, in particular iron deficiency anaemia (IDA), present a considerable burden on health in developing countries (WHO 2011).

Given the predominance of IDA in the world-wide prevalence data (WHO 2015), it is therefore important to direct research efforts to improve the diagnosis and management of this ailment. The diagnosis of medical conditions has always required specialised skill and professional judgement, and this applies to haematological disorders such as iron deficiency anaemia as well. Research aimed at improving diagnostic sensitivity of iron deficiency anaemia is well placed and this investigation has been directed at exactly that. Increased diagnostic sensitivity would result in earlier detection of the disease in question, in this instance IDA. Once diagnosed earlier, treatment can be initiated much sooner as well. For IDA the treatment is simple and has a high success rate given that IDA is a nutritional deficiency disorder.

The diagnosis of IDA relies on confirming the presence of anaemia by determining the level of haemoglobin and detecting the presence of inadequate iron bioavailability (Thomas et al., 2013). Inadequate iron bioavailability refers to a state whereby there is not enough iron for normal physiological functions such as erythropoiesis.

When there are insufficient iron supplies for erythropoietic activity the body invokes



compensatory mechanisms. These mechanisms would result in altered red blood cell shape and haemoglobin composition and in due course would result in anaemia. Inadequate iron bioavailability can be detected using a variety of approaches which include presence of distinctive red cell morphological characteristics indicative of iron underutilization and the presence of abnormal red cell indices obtainable from a full blood count such as mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC). Inadequate iron bioavailability can also be detected through biochemical analysis of blood samples to confirm the presence of abnormal iron studies results. Iron studies encompass serum ferritin, serum iron, total iron binding capacity and transferrin levels. A more invasive approach would be to collect a bone marrow sample and look for the presence of stainable iron deposits. This is done by staining a bone marrow aspirate with a pearls Prussian blue stain. A bone marrow biopsy is not usually required in this instance unless there is a failed aspirate or bone marrow fibrosis is suspected.

Current UK guidelines on diagnosis of iron deficiency anaemia suggest the use of Full blood count results and serum ferritin levels (Thomas et al., 2013). Full blood count generates a battery of parameters which have to be interpreted in conjunction with another set of results for iron studies, a situation which complicates the decision making process. In Namibia, the Namibia standard treatment guidelines (MoHSS 2011) informs the approach used to diagnose iron deficiency anaemia. The Namibian guidelines rely on the findings of a low MCV, low MCHC together with a microcytic hypochromic blood picture in order to suspect iron deficiency anaemia.

The current approaches do not make use of Hepcidin as a routine diagnostic tool for iron deficiency anaemia but Girelli et al., (2016) and other authors have highlighted its potential. Pasricha et al., (2013) has even advocated for Hepcidin point of care testing (POCT) as a research priority area in looking for ways to manage post malarial iron deficiency anaemia. According to Kroot et al., (2011) the measurement of Hepcidin is still a promising diagnostic tool, especially for the diagnosis and management of medical conditions in which iron metabolism is affected such as iron deficiency anaemia. The role of Hepcidin in directly regulating iron makes it a relevant marker of iron bioavailability. Hepcidin is sensitive to the dynamics of iron utilisation and could

potentially be used as a routine laboratory test to help in the diagnosis and management of patients with iron deficiency anaemia. A reduction in iron bioavailability results in reduced Hepcidin levels due to its suppressed expression. These features have resulted in the current UK guidelines (Thomas et al., 2013) alluding to the horizon of Hepcidin as potential marker in iron deficiency anaemia.

### **1.1. Research aims**

The aims of this study are to investigate how hepcidin 25 (referred to as Hepcidin in this study) can be introduced into the mainstream routine diagnosis laboratory. The study intended to investigate how Hepcidin can be used as a more sensitive routine diagnostic test for patients presenting with iron deficiency anaemia in Namibia, a country where the prevalence of anaemia in general is around 40% (de Benoist et al., 2008). This research project intended to achieve this by establishing quantitative differences of hepcidin 25 (Hepcidin) molecules in participants with features suggestive of iron deficiency anaemia. The study capitalises on the established relationship between iron and Hepcidin and thus looked at iron deficiency anaemia in Namibia using participants with features suggestive of iron deficiency anaemia. These features include reduced haemoglobin levels which correct when iron is introduced. Correction of anaemia post iron treatment being regarded as a confirmation for the presence of iron deficiency anaemia at initial presentation. In an attempt to come up with baseline Hepcidin values, the study worked on establishing Hepcidin reference normal ranges for the Namibian population as a starting point before any work on IDA was done.

### **1.2. Specific objectives**

The specific objectives for this study were:

- To establish the reference normal range for Hepcidin in a healthy adult population in Namibia where healthy adult is defined as: participants between 16 years and 65 years; haemoglobin >125g/L and eligible blood donors. The NaMBTS blood donation criteria was useful in defining “normal healthy adult”
- To assess the values of Hepcidin in iron deficiency anaemia in Namibia by measuring serum hepcidin 25 (Hepcidin) concentrations in participants who are iron

deficient.

- To characterise the quantitative behaviour of Hepcidin in participants receiving iron treatment. The quantitative behaviours of Hepcidin were characterised by assessing its correlations and independent associations with serum iron and full blood count parameters such as red cell indices and haemoglobin levels.

### **1.3. Overview of experimental methods**

The study used an ELISA based Hepcidin assay to quantitate hepcidin 25 levels in three groups of participants namely reference normal range (RNR), pre-iron treatment iron deficiency anaemia group (Pre-Iron) and post iron treatment iron deficiency anaemia group (Post Iron). Full blood count and serum iron analysis was also performed on the same three study groups i.e. RNR, Pre-Iron and Post Iron. The full blood count analysis was carried out using ABX Pentra XL80 analyser. Serum iron assays were manually set up using the FerroZine iron kit manufactured by Chrono lab systems, SPAIN.

Research on the first group has been aimed at establishing reference normal ranges while work on the second and third groups was devoted to characterizing the behaviour of Hepcidin in iron deficiency anaemia. The three groups were: 1) healthy adults, 2) individuals with features suggestive of iron deficiency anaemia pre-iron treatment and 3) individuals with features suggestive of iron deficiency anaemia post iron treatment. Correlation studies of current tests and Hepcidin assays were performed on participants from the groups being studied. In this study, collection of dual point data sets from patients on iron therapy was carried out by measuring serum iron, Hepcidin and full blood count parameters on IDA patients before and after treatment with iron. The data obtained was used to assess the potential usefulness of Hepcidin as a more sensitive biomarker in such clinical situations.

### **1.4. Benefits of a successful outcome of research**

This research work looked at the usefulness of Hepcidin from a diagnostic point of view. In their review, Kroot et al., (2011) alluded to the fact that Hepcidin measurements may be useful in anticipating the fate of iron therapy in associated diseases such as iron deficiency anaemia. This research therefore worked towards making more information available for clinicians by giving them further insight into

what happens to iron regulation by Hepcidin in patients who have iron deficiency anaemia.

Establishment of the usefulness of Hepcidin assays if proved useful in clinical settings as described above may reduce the lead time to decision making and may also shorten patients' hospital stay. The benefits of Hepcidin testing will go beyond reducing lead-time to decision making due to its possible increased diagnostic sensitivity. It can also be used to manage IDA patients requiring simple routine surgical procedures by informing the surgical teams of the likely outcome of blood loss in terms of subsequent erythropoietic activity. Hepcidin levels above the lower limit of normal indicate supposedly adequate iron bioavailability and consequently competent erythropoietic compensation. On the other hand reduced Hepcidin levels signal its suppression as a result of iron bioavailability. The extent of reduced Hepcidin levels and its relationship with levels of erythropoietic activity was part of this research whose findings play an important role in elucidating this relationship further.

## CHAPTER 2

### 2. LITERATURE REVIEW

#### 2.1. Anaemia

Anaemia is the most common red cell disorder and it occurs when the concentration of haemoglobin (Hb) falls below normal levels for a person's age and gender (Hoffbrand and Moss 2011 2010). The World Health Organization (WHO) defines anaemia as a decrease in the concentration of Hb and sets the haemoglobin thresholds at <130 g/L and < 110 g/L for adult males and non-pregnant females respectively (WHO 2011). The threshold as to what constitutes a reduced Hb varies according to gender, age and geographical location. In view of this, the World Health Organization also defines anaemia as Hb concentration below 110 g/L in pregnant women, 110g/L in children under five and 115g/L in older children (WHO 2011). The thresholds for defining anaemia in Namibia are different to those quoted by the WHO because of the variations in geographical locations. In Namibia anaemia is defined on the basis of normal ranges as defined by the South Africa Institute of Medical Research (SAIMR) reference ranges for Haematology in the Namibia Standard Treatment Guidelines (Ministry of Health and Social Services 2011). It is defined as a haemoglobin value less than 143 g/L and 121g/L for adult males and non-pregnant females respectively (MoHSS 2011).

A reduced haemoglobin (Hb) level is the hall mark of anaemia. In addition, other haematological values relevant to anaemia include Red Blood Cell Count (RBC), Red cell Distribution Width (RDW), haematocrit (HCT) as well as other red cell indices such as Mean Corpuscular volume (MCV), Mean Corpuscular Haemoglobin (MCH) and Mean Corpuscular Haemoglobin Concentration (MCHC). These additional parameters are useful in elucidating the different types of anaemias, in particular iron deficiency anaemia (IDA). Iron deficiency anaemia is a nutritional disorder affecting red cell production as a result of reduced iron bioavailability. Pasricha et al., (2013) state that treatment for IDA is simple, safe and effective. It is therefore prudent that accurate diagnosis is made to enable proper management of this

ailment. According to Pasricha et al., (2013), in 2004 WHO estimated that IDA resulted in 273 000 deaths of which 31% of them were in Africa. Given the ease with which IDA can be managed, the importance of its diagnosis cannot be emphasized enough.

Red cell indices such as MCV, MCH, MCHC and RDW give useful diagnostic information in IDA. A low haemoglobin value has a direct impact on the mean corpuscular haemoglobin (MCH) and the mean corpuscular haemoglobin concentration (MCHC) because these are indices derived from the Hb value and other red cell parameters. A mean corpuscular volume (MCV) is however a direct reflection of the average cell size of the red blood cells. This is reduced in iron deficiency anaemia as a physiological response to reduced iron bioavailability and consequently reduced haemoglobin (Hoffbrand and Moss 2011). During normal erythropoiesis, red blood cells are packaged with adequate amounts of haemoglobin. This however is not the case when there is insufficient iron.

The red cell distribution width (RDW) gives an indication of how variable the cells are, and it is a good indicator of the extent of anisopoikilocytosis. This value is increased in iron deficiency anaemia because of the presence of microcytic red blood cells as well as other abnormally shaped cells such as pencil cells. Pencil cells are a common feature in iron deficiency anaemia (Provan 2007) to such an extent it has become a diagnostic morphological feature for this pathology. In their work, Sazawal et al., (2014) presented convincing results to motivate the use of only RDW and Hb, without the use of iron studies results, in the diagnosis of iron deficiency anaemia. The merit of this approach is however debatable given the fact that the RDW is only an indicator of red cell variability.

## **2.2. Iron deficiency anaemia**

Iron deficiency anaemia is a condition with vague clinical presentation. It is a condition which is characterised by low iron bioavailability for normal physiological functions (Hlatswayo et al 2016). The reduced iron bioavailability results in restricted haemoglobin production leading to a detectable anaemia. Anaemia upregulates the production of erythropoietin, a hormone which stimulates red cell production. However, in iron deficiency, increased erythropoietin production does not correct the anaemia even

when red cell production is stimulated. This is due to the absence of iron, a major component for haemoglobin synthesis.

Iron deficiency anaemia is a serious health problem that affects more than 2 billion people globally (WHO 2015). It is more prevalent in developing countries where nutritional deficiencies are rampant. The 2011 global prevalence data on anaemia indicated that more than 40% of all anaemia cases is attributed to iron deficiency (WHO2015). In Namibia a country among the Africa sub Saharan countries, the prevalence is even higher. WHO global data base on anaemia estimates the prevalence to be about 60% for Namibia (WHO 2008). Given the prevalence data on this disease it is important to pay attention on both its diagnosis and treatment. Furthermore, IDA has been estimated to cause about 800 000 deaths and a loss of more than 35 million disability adjusted life years worldwide (WHO/CDC 2005). These reports strengthen the case for efforts to focus on IDA especially from diagnosis, treatment and prevention points of view.

Iron is required on a daily basis to compensate for losses from the body via sloughing of iron replete intestinal cells, bleeding and menstruation. The amount of replacement iron required varies from 0.5 milligrams to 2 milligrams depending on age and gender (Hoffbrand and Moss 2011). Iron deficiency occurs when the body fails to replace the required amounts, and this can be due to various factors discussed in section 2.2.1.

#### **2.2.1. Causes of iron deficiency**

Iron deficiency occurs when demand exceeds supply, and this can be due to various factors. In developing countries nutritional deficiency is a major cause of iron deficiency (WHO 2015) as well as hookworm infestation and *Helicobacter pylori* infection (Hoffbrand and Moss 2011). In nutritional deficiency, iron bioavailability is reduced even when the demand remains the same. On the contrary, reduced iron bioavailability in hookworm infestation or *H. Pylori* infection is due to increased demand. Other causes of iron deficiency also include blood loss, alcoholism and increased physiological demand such as in pregnancy.

Nutritional deficiencies occur due to consumption of foods of low nutritional value and

this is common in developing countries where hunger and malnutrition are a common occurrence (Bailey et al., 2015; de Benoist et al., 2008; WHO 2011). Foods rich in iron include meat, fish and poultry products which are relatively expensive. Such foods are also not a priority when financial resources are constrained. Furthermore, animal and poultry products do not form part of a staple diet in developing countries where communal farming is the main form of income and livelihood. This causes IDA usually occur over time because iron stores are gradually reduced due to an imbalance in iron uptake and iron loss.

Parasitic infestation causes iron deficiency due to increased demand on iron because the parasites feed on the human body. The parasites in particular hookworms reside in the gastrointestinal tract which encourages sloughing of the cells they come into contact with. Obscure occult bleeding as well as gastrointestinal bleeding are common features in hookworm infestation. In such instances, iron is lost as haemoglobin contained in intact red cells. Blood loss in general causes iron deficiency because most of the iron in the body is depleted in trying to compensate for the blood loss.

### **2.3. Impact of iron deficiency anaemia**

The effects of IDA on general health are wide and diverse with consequences that can span an individual's entire life. Even though IDA is a nutritional disorder that can be transient, its consequences can be perpetuated across generations (Bailey et al., 2015). It is therefore imperative to manage this ailment as soon as it is diagnosed to prevent long-reaching untoward effects that can emanate from this disorder. The effects include impaired cognitive development, decreased productivity, and increased maternal mortality amongst other consequences.

#### **2.3.1 Functional consequences of iron deficiency anaemia**

Functional consequences are defined as the alteration of an individual's actual or potential capacity to carry out tasks that are regarded as normal for their age (Kirch 2008). Functional ability is influenced by cognitive development, muscle function and physical performance.

An association between IDA and impaired cognitive development has been reported



(Black 2014). The Global Burden of Disease (2000) project confirmed that iron deficiency contributes to impaired cognitive development (Stoltzfus et al 2003). Other studies by Beltran-Navarro and their team concluded that the development of environmental sound perception and motor skills is affected by iron deficiency (Beltran-Navarro et al 2012). This is explained by the role of iron in brain development as enzymes that contain iron are involved in myelination of the neurons and formation of dendrites. The lack of iron disturbs the dendritogenesis and consequently neuronal development. The effect of iron deficiency in neuronal development is irreversible resulting in life long effects of impaired cognitive development such as low academic performances, anxiety and depression (Hlatswayo et al 2016).

It has also been established that iron deficiency has a negative impact on an individuals' productivity at work (Stoltzfus et al 2003). This is mainly due to the effect IDA has in reducing energetic efficiency and endurance levels. In separate studies, Denvir et al (2015) established a linear relationship between iron deficiency and physical activity. With decreased physical activity reduced productivity can be inferred especially in workplaces where physical activity is imperative such as in production factories.

### **2.3.2 Maternal mortality in iron deficiency anaemia**

Two meta-analyses drawing upon the same published studies reported on an association between IDA and maternal mortality. Brabin et al (2001) suggested that there is an association between a higher risk of maternal mortality with severe anaemia. Stoltzfus and colleagues concurred with these findings suggesting that the risk of maternal mortality and haemoglobin levels have a non-linear relationship (Stoltzfus et al., 2005).

A negative correlation between maternal IDA and gestation period is also well established (Allen, 2001). There are currently two widely accepted biological mechanisms that explain this phenomenon. One of the theories suggests that anaemia causes hypoxia to the unborn foetus. It further suggests that anaemia induces maternal and foetal stress leading to production of corticotropin-releasing hormone (Allen, 2001).

Maternal iron deficiency is strongly associated with low birth weight and stunted growth (Stoltzfus et al., 2005). These effects are evident regardless of the presence or absence of anaemia. It is also pertinent to note that while full term infants are normally born with sufficient iron stores they may develop iron deficiency after birth. This is because infants have high iron requirements and the diets offered to them do not usually meet this demand.

### **2.3.3. Implication of iron deficiency on red blood cell production.**

Iron is required for red blood cell production where it is incorporated into the haem molecule. The haem moiety combines with a globin chain, four of which make up a haemoglobin molecule which is the oxygen carrier molecule for red blood cells.

During haem synthesis glycine combines with succinyl coenzyme A to form porphobilinogen. This biochemical reaction is catalysed by  $\delta$  aminolaevulinic acid ( $\delta$  ALA). Porphobilinogen goes through a series of biochemical reactions in the cytoplasm before returning to the ribosomes as protoporphyrin. Protoporphyrin combines with ferrous iron to form a haem molecule (Hoffbrand and Moss 2011). From this discussion, it is evident that reduction in iron levels has a direct impact on haem synthesis and subsequently haemoglobin production. In its initial stage, iron deficiency result in formation of red blood cells with reduced amounts of haemoglobin. These are referred to as hypochromic red blood cells. In conjunction with hypochromasia, the red cells are also smaller than normal and are referred to as microcytes. These features are present even before anaemia becomes evident.

The production of microcytic hypochromic red blood cells is a compensatory mechanism to try and maintain normal erythropoietic activity in the absence of adequate iron supply. If the condition is not corrected, progressive decline in haemoglobin production occurs and eventually the patient becomes anaemic. Other red cell morphological features such as pencil shaped cells and target cells pursue. The formation of these abnormal red cell morphologies shortens the life span of the cells which further ameliorates the anaemia (Kempe et al., 2006) Abnormal red blood cells are removed and destroyed by the reticuloendothelial cells as the red blood cells pass through the spleen and other reticuloendothelial systems.

## **2.4 Diagnostic strategies for iron deficiency anaemia**

The common strategies for diagnosing iron deficiency anaemia relies on the detection of low levels of bioavailable iron and decreasing haemoglobin levels. The amount of available iron can be assessed in different ways some of which are invasive. Haemoglobin determination however is usually carried out by performing a full blood count. A full blood count result is useful in determining haemoglobin levels as well as other parameters that are of diagnostic significance.

### **2.4.1. Full Blood Count as an indicator of Iron deficiency anaemia.**

In Iron Deficiency Anaemia (IDA) a full blood count test result would show a reduced haemoglobin level below the set threshold for age and gender.

Apart from a haemoglobin value, other parameters such as red cell indices and red cell morphological features are useful in diagnosing IDA. Relevant red cells indices are Mean Cell Haemoglobin (MCH), Mean Corpuscular Haemoglobin Concentration (MCHC), and Mean Corpuscular Volume (MCV). These three parameters give an indication of cell size and level of haemoglobinisation. The following ranges are regarded as normal for an adult:

MCV: 80 - 95 femtoliters

MCH: 27 - 34 picograms/cell

MCHC: 300 - 350 grams/litre. (Hoffbrand and Moss, 2011)

These values are a guide and every geographical location and laboratory usually establishes their own ranges suitable for the populace they serve. An MCV below the lower limit of normal gives an indication of microcytosis and this is usually encountered in IDA and certain haemoglobinopathies. An MCHC below the lower limit of normal gives an indication of hypochromasia which again is an indication of inadequate haemoglobin and hence inadequate iron bioavailability. In this case Hepcidin values would give a better indication of iron status compared to MCHC values however Hepcidin is not part of a full blood count result. Mean corpuscular haemoglobin is a good indicator of hypochromasia and hence IDA. However, Thomas et al., (2013) recommends the use of percentage hypochromic cells as a better indicator.

From the FBC results, a diagnosis of microcytic hypochromic anaemia can be made. A peripheral blood film however needs to be examined in order to be more certain that IDA is present unless there is supporting clinical information. Common features for IDA that are found on a peripheral blood film include pencil cells and target cells.

#### **2.4.2. Iron levels as an indicator of Iron Deficiency Anaemia (IDA)**

Availability of iron is related to the presence of iron deficiency anaemia in all but a few cases. This is explainable by the fact that iron is essential in the early stages of erythropoiesis when it is incorporated to the haem molecule. For this reason, assessment of iron levels in the bone marrow has been made the gold standard test for iron deficiency anaemia (Johnson-Wimbley and Graham 2011). Other indicators of iron bioavailability include serum iron levels, serum ferritin levels as well as total iron binding capacity. Ferritin is the storage form of iron and a reduction in this parameter indicates reduction in stored iron and subsequently reduced iron bioavailability. Ferritin levels may however be misleading because it is affected by inflammation. Inflammatory conditions result in an increase in ferritin levels because it is an acute phase reactant (Clark 2009). The measurement of serum iron gives a better picture as it assesses the presence of both iron and ferritin when using the Ferrozine colourimetric method (CHRONOLAB n.d.). This method allows for iron in transferrin to dissociate first before being assayed together with other non-bound iron.

#### **2.4.3. Presence of stainable iron as a measure of iron bioavailability**

The gold standard method for assessment of iron bioavailability includes the demonstration of stainable iron in the bone marrow. This could be either a bone marrow aspirate or trephine biopsy but in both cases obtaining the sample is invasive and labour intensive. The bone marrow sample is stained with an iron stain such as Pearls Prussian blue stain. In iron deficiency anaemia little or no demonstrable iron is present in the bone marrow (Hoffbrand and Moss 2011). A closer look at the developing erythroblasts also show absence of iron and the presence of smaller than normal erythroblasts which will eventually give rise to microcytic red blood cells.

## **2.5. Overview of the current diagnostic strategies for IDA.**

Generally diagnostic approaches are informed by guidelines and IDA is not an exception. Guidelines help to standardise the diagnostic approaches and they take cognisance of both current evidence as well as the cost implications of all the available options.

### **2.5.1. Current guidelines**

Guidelines on diagnosis of anaemia rely on clinical symptoms, medical history as well as laboratory results. Clinical symptoms for anaemia in general include fatigue, loss of energy, pale skin and shortness of breath which is more evident during exercise (Hoffbrand and Moss 2011). All these are a result of a reduction in the red blood cells' oxygen carrying capacity brought about by a reduction in haemoglobin level or function. Medical history for a patient with anaemia will include lack of concentration and feeling tired all the time. These findings usually tie up with the clinical symptoms, but laboratory investigations are imperative for a diagnosis of anaemia, in particular iron deficiency anaemia, to be confirmed.

Guidelines on laboratory diagnosis of iron deficiency anaemia vary in different parts of the world. They are however based on the same principles of observing the haemoglobin values with lower values being used to define anaemia. The threshold for defining anaemia however varies depending on locally agreed reference normal range values. In fact, Odhiambo et al., (2015) and Huma & Waheem (2013) commends that the establishment of local reference normal ranges is important in countering physiological variations due to differing demographics

The presence of iron deficiency can be assessed in different ways, the most common of which are low serum iron values, depressed ferritin levels in serum, reduced iron stores in the bone marrow and red cell morphological features associated with erythropoiesis in iron deplete states. In reduced iron levels, smaller than normal red cells are produced and inadequately haemoglobinised red blood cells are a common feature. The presence of pencil shaped red cells is also synonymous with iron deficiency. Pencil cells, together with the presence of varying populations of red cell sizes gives rise to higher than normal red cell distribution width which again is

a pointer to iron deficiency anaemia.

### **2.5.2. Namibian approach to diagnosis of iron deficiency**

Diagnosis of iron deficiency anaemia can either be straightforward or it could be marred by the presence of underlying medical conditions. Common medical conditions associated with iron deficiency anaemia include pregnancy and malnutrition. Pregnancy presents an increased demand due to the nutritional needs of the developing foetus. Malnutrition results in reduced intake of iron among other nutrients. In all scenarios, the diagnostic approach for IDA is the same according to the Namibian treatment guidelines. There are however differences in clinical presentations with some concurrent medical conditions masking the clinical features of IDA thus the treatment guidelines highlight the importance of laboratory testing, medical history and physical examination (MoHSS 2011).

The diagnosis of anaemias in general is also guided by the Namibia standard treatment guidelines (MoHSS 2011). Anaemia in this case is defined as a haemoglobin level below the lower limit of reference normal range and these ranges differ according to age and gender (MoHSS 2011). Low haemoglobin levels and low Mean Corpuscular Volume (MCV) values indicate microcytic hypochromic anaemia of which iron deficiency anaemia belongs to this group of anaemias. Other microcytic hypochromic anaemias include haemoglobinopathies in which case a differential diagnosis is needed before iron deficiency anaemia is confirmed. In light of this, the Namibia standard treatment guidelines (MoHSS 2011) state that a full blood count should be done, and red cell indices looked at. Iron deficiency anaemia should be suspected if the MCV is low and further testing initiated to confirm its presence.

The guidelines further suggest the use of a peripheral blood smear as well as iron studies to confirm the presence of iron deficiency (MoHSS 2011). Iron studies assess physiological iron bioavailability and peripheral blood smears assess the presence of morphological features associated with iron deficiency anaemia. These parameters complement each other when carrying out a diagnostic workout for iron deficiency anaemia. These guidelines are in line with the UK guidelines on the diagnosis of anaemia. The UK guidelines suggest the use of the same parameters

including red cell indices such as MCV and Mean Corpuscular Haemoglobin Concentration (MCHC) (Thomas et al., 2013).

This approach, although it works, can be improved by the introduction of Hepcidin testing. Hepcidin testing can complement other informative tests such as haemoglobin levels as well as replace other invasive tests that are used to assess presence of adequate amounts of iron. Invasive tests such as assessment of bone marrow iron stores and expensive tests such as iron studies could be streamlined once Hepcidin is introduced as a routine diagnostic test for the diagnosis and prognosis of IDA. Iron studies are a battery of tests which include serum ferritin, serum iron, transferrin and total iron binding capacity. The general shortfalls of the current approach include the need for a battery of tests to make a diagnosis for a simple disorder such as iron deficiency anaemia. The need for invasive tests such as bone marrow biopsy to assess iron stores also makes it an expensive diagnostic approach requiring not only the laboratory staff but also clinical haematology staff who are, in most cases, consultant grade medical staff.

### **2.5.3. Proposed approach**

With the introduction of Hepcidin testing it may be possible to streamline the diagnostic tests needed to identify iron deficiency anaemia. A full blood count result and Hepcidin test could be all that is needed for both the diagnosis and prognosis of IDA. A full blood count result gives parameters such as haemoglobin which confirms the presence of anaemia. Other parameters, namely red cell indices such as Mean Corpuscular Volume (MCV) confirms microcytosis. Hepcidin can therefore be used to elucidate the cause of microcytic anaemia. Hepcidin levels below the lower limit of normal in a patient without medical history of chronic disease should be able to confirm IDA. Hepcidin levels are suppressed in IDA and this study seeks to assess the magnitude of the level of suppression. In so doing Hepcidin levels from a reference normal range group were established and used to assess how the degree of Hepcidin suppression varies in IDA.

Patients on treatment for IDA usually require a correction in red cell indices especially MCV to ascertain treatment success. Hepcidin has an immediate effect on iron levels (Rivera et al., 2005) in turn it is also more sensitive to iron bioavailability. It therefore is

a more sensitive prognostic marker. Therefore, IDA patients on iron treatment who demonstrate rising levels of Hepcidin should be considered as a positive outcome. This should be considered a treatment success even without evidence of repleted iron stores.

Hepcidin could reduce lead time to diagnosis once a model is established that relates the dynamics of Hb with changes in Hepcidin levels. This will consequently make it a more sensitive prognostic indicator. Efforts to expand the current knowledge base on Hepcidin levels are therefore essential for this ultimate goal. This research work focuses on establishing normal ranges in the Namibian population as well as differentiating iron deficiency from non-iron deficient states using Hepcidin.

In their work (Abosakran et al., 2014) established the correlation between red cell count and Hepcidin as well as haemoglobin and Hepcidin in a paediatric population. Takasawa et al., (2015) also performed correlation studies with MCV and Hepcidin  $r=0.20$  albeit with a higher p value of 0.1655 which meant the results were not statistically significant. The same correlations can be done with other RBC indices. In this current study, correlations between Hb and Hepcidin were established for both the normal reference ranges population as well as for the IDA population. Correlations for Hepcidin with MCV, RDW as well as MCHC were also established with the intention of finding out more appropriate indices to use. The relationship between Hb levels and earlier Hepcidin concentration was also used to investigate the utility of Hepcidin in reducing lead time to diagnosis. Correlations between Hepcidin levels and change in haemoglobin concentrations ( $\Delta\text{Hb}$ ) were also established in an attempt to strengthen the case for using Hepcidin as a predictor of response to iron supplementation.

## **2.6. Introducing Hepcidin**

Hepcidin was first discovered in 2000 by Krause et al., (2000) in human blood ultra-filtrate and urine samples (Ganz 2003; Kemna et al., 2008; Politou et al., 2004). It was originally called liver-expressed antimicrobial peptide (LEAP-1) because of its antimicrobial activity against some gram-positive *Bacillus* and *Staphylococcus species* as well as a *Neisseria species*, *Neisseria cinerea* which is a gram-negative bacterium (Krause et al., 2000). The anti-microbial activity of Hepcidin goes beyond the bacterium kingdom; it also shows anti-fungal activity against *Saccharomyces*



cerevisiae, commonly known as brewer's yeast. Tavanti et al., (2011) also demonstrated the anti-fungal effect of Hepcidin albeit Hepcidin 20, against *Candida* species and this goes on to show its broad antimicrobial properties over and above its role in iron metabolism. Since its discovery and the unearthing of its antimicrobial activity, it took a further year to confirm the existence of the link between Hepcidin and iron metabolism (Pigeon et al., 2001). The linkage forms the basis of this research work which looked at the behaviour of Hepcidin with reference to iron's involvement in red blood cell production. The Hepcidin-iron linkage also further clarified the relationship between Hepcidin and some of the haematopoietic products such as red blood cells.

Since its discovery, a lot of work has gone into characterising this molecule. The name 'hepcidin' originated from the anatomical site of its synthesis as well as its anti-microbial activity (Politou et al., 2004). The prefix hep-; is derived from the word hepatocytes which are liver cells and the suffix-cidin which is usually designated to antibiotics, emanates from the antimicrobial activity of the molecule. Hepcidin is synthesized mainly in the liver as pro-hepcidin, an inactive form of hepcidin (Ganz 2011). Other organs have been known to produce hepcidin as well and these include the kidneys (Kulaksiz et al., 2005) and the heart (Isoda et al., 2010).

Once produced, pre-prohepcidin is firstly converted to an inactive pro- hepcidin polypeptide which is then converted to an active Hepcidin molecule by a pro protein convertase enzyme called furin which cleaves it into a smaller molecule (Poli et al., 2014; Ganz et al., 2011). The smaller molecule is the active form of the hormone, hepcidin. Hepcidin is a polypeptide existing in three main isomer forms namely: hepcidin 25; hepcidin 22 and hepcidin 20. The isomers have 25, 22 and 20 amino acids respectively (Kenma et al., 2008).

Hepcidin 25 and hepcidin 20 are detectable in human serum but all three isomers are present in urine samples (Campostrini et al., 2010; Kenma et al., 2008). Hepcidin 25 is the major form of hepcidin responsible for iron regulation despite the presence of several isomers. Hepcidin 20 contains greater antimicrobial properties than hepcidin 25 but does not have the ability to degrade ferroportin (De Domenico et al., 2008) a major drawback to its ability to regulate iron. This leaves hepcidin 25 as the major

regulator of Iron detectable in human blood.

Formation of hepcidin is under the control of the hepcidin antimicrobial peptide (HAMP) gene which is located on chromosome 19q13 (Kenma et al., 2008). Its expression however is under the influence of many factors which include hemojuvelin (Papanikolaou 2004) High iron Fe (HFE) protein (Ahmad et al., 2002) and the Bone Morphogenic Proteins (Truksa et al., 2006). These factors play an important role in the signalling pathways that take place for hepcidin expression and they are discussed in section 2.6.1 below.

### **2.6.1. Hepcidin signalling pathways**

Cell signalling pathways are indistinct, at times their activities depend on other pathways and this makes them even more complex. They do not function independently; instead they constitute complex pathways with which the effects of initial interactions are amplified on the next stages (Hancock 2010). At present only two iron-related signalling pathways that are responsible for the expression of Hepcidin have been established and these involve the Bone Morphogenic Proteins (BMP) and the Transferrin Receptors (TFR) respectively (Knutson 2010). These two signalling pathways independently upregulate Hepcidin expression when activated. There is however evidence to suggest that there is crosstalk that exists between the BMP and TFR signalling pathways. This demonstrates the high level of complex interactions that occur in these distinct signalling pathways.

The BMPs regulate Hepcidin expression via the bone morphogenic proteins/hemojuvelin/sons of mothers against decapentaplegic (BMP/HJV/SMAD) signalling pathway. BMPs are a group of about 20 molecules with hormonal properties and they include BMP1 to BMP15 with BMP6 being the key regulator of Hepcidin although this is yet to be demonstrated with human subjects (Andriopoulos et al., 2009; Meynard et al., 2009). Babitt et al., (2007) and Truksa et al's (2006) work on BMPs established the link between the BMPs and Hepcidin expression. They were able to demonstrate that BMPs2, 4 and 9 are also able to regulate Hepcidin expression notwithstanding the fact that BMP6 is the main protein involved. Meynard et al's (2009) work which proved that systematically destroying BMP6 in mice resulted in

undetectable levels of hepcidin also confirmed that BMP is a key regulator of hepcidin expression.

From the studies, it was also possible to infer that the BMP/HJV/SMAD pathway regulate hepcidin expression in response to iron bioavailability. Figure 2.1 illustrates the BMP/HJV/SMAD pathway.

**Figure 2.1: The BMP/HJV/SMAD pathway and the role of BMP receptors. Reproduced from: Muckenthaler M. U. (2014). How mutant HFE causes hereditary hemochromatosis *Blood* 124 (8):1212-1213**

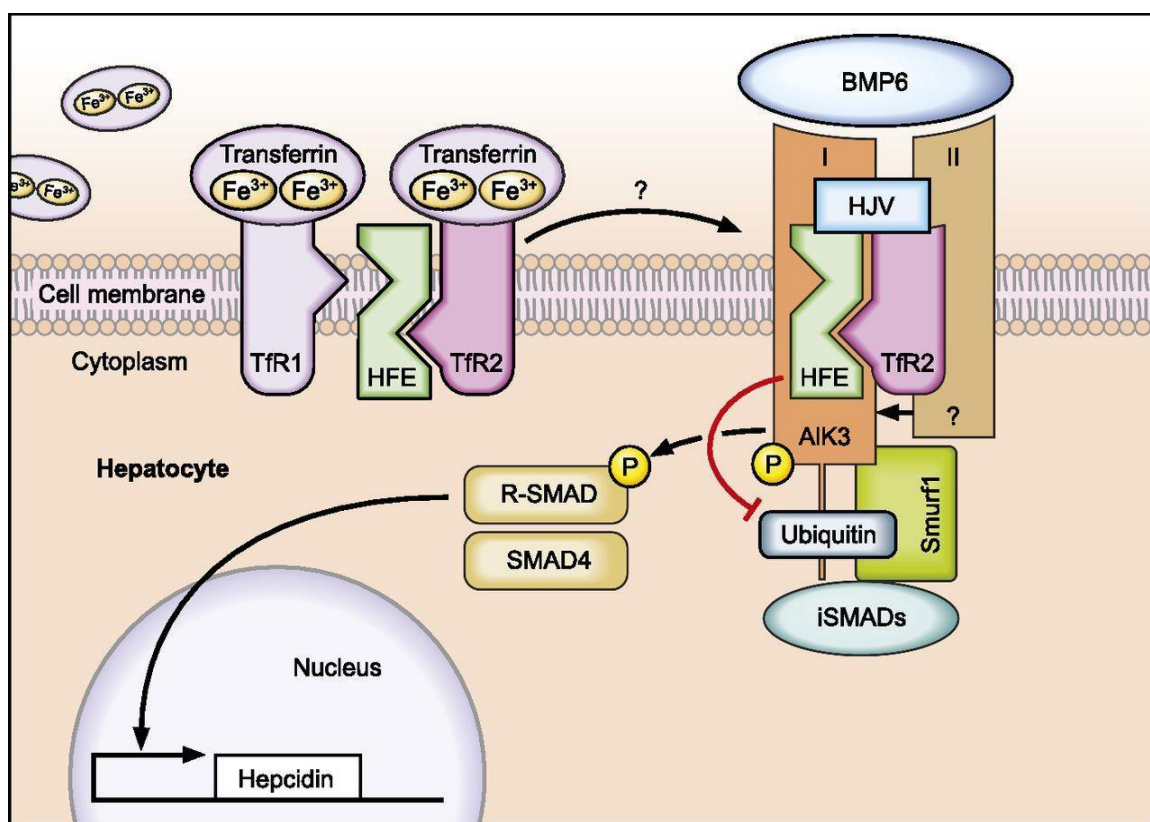
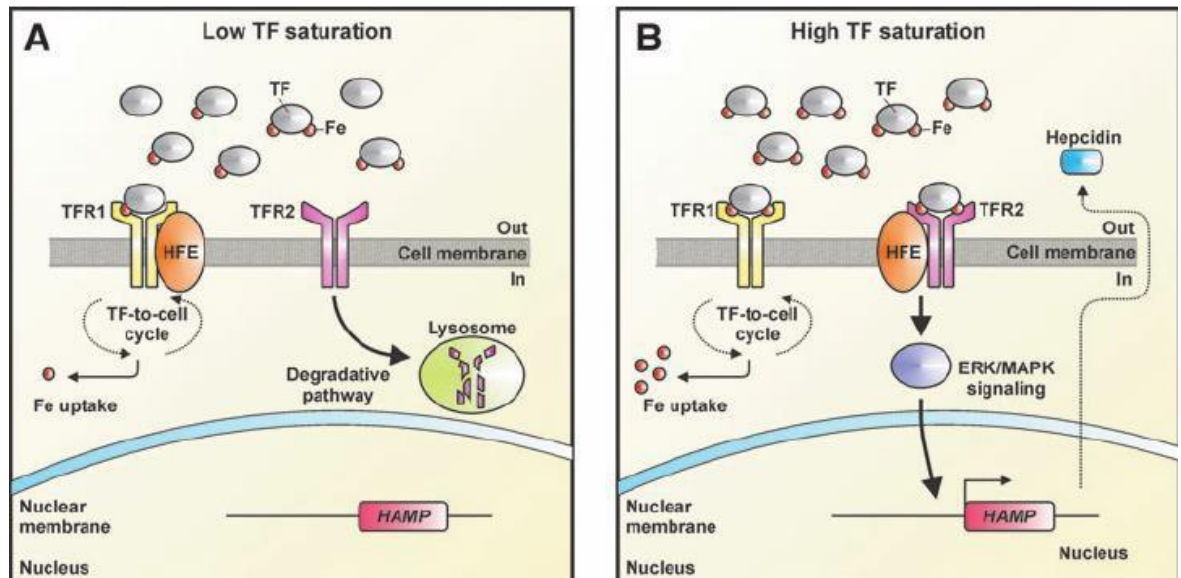


Figure 2.1 above shows how hepcidin expression is regulated by the interactions between TFR, Hfe, HJV and SMAD proteins. Hfe interacts with TFR2 to form a signalling complex together with HJV. The Hfe/TFR2/HJV complex binds to BMP receptors (labelled as I and II on the diagram in Figure 2.1) to avert being destroyed. The binding of the Hfe/TRF2/HJV complex to BMP receptors results in the increased presence of the BMP receptor on the cell surface. The BMP receptors allow for BMPs in particular BMP6 to attach thereby activating the signalling of hepcidin transcription by BMP/SMAD.

The BMP/HJV/SMAD signalling pathway can either be mediated by inflammation or iron, the latter being the relevant one in this study. In the iron mediated BMP/HJV/SMAD pathway, BMP6 binds to BMP receptors on the surface of the cell membrane. This interaction increases the phosphorylation of SMAD proteins which then translocate to the nucleus to upregulate transcription of Hepcidin (Core et al., 2014; Mieczko-Sanecka et al., 2014). The interaction of the bone morphogenetic proteins with its receptors requires haemojuvelin as a co-receptor hence the name BMP/HJV/SMAD signalling pathway.

The other major signalling pathway for Hepcidin is the Transferrin Receptors (TFRs) dependent signalling pathway which is illustrated in Figure 2.2. In this pathway the amount of available iron has an effect on whether Transferrin receptor 2 (TFR2) complexes with holotransferrin or not. When there is low iron bioavailability, the amount of holotransferrin is reduced and consequently holotransferrin-TFR2 complexing is minimal. Inversely, when there are increased levels of holotransferrin its complexing with TFR2 is increased and so is the extracellular signal-regulated kinases/mitogen activated protein kinase (ERK/MAPK) signalling.

**Figure 2.2: Role of Transferrin receptors in regulating Hepcidin expression in iron deplete (A) and iron replete (B) states. The TFR/MAPK signalling pathway as illustrated by Lawen A., Lane D. J. R. 2013. Mammalian Iron Homeostasis in Health and Disease: Uptake, Storage, Transport, and Molecular Mechanisms of Action *Antioxidants & Redox Signalling*: 18 (18): page 2489**



In the TFR pathway, binding of TFR2 with Hfe results in the phosphorylation of mitogen activated protein (MAP) kinases and their translocation to the nucleus. Once they reach the nucleus these activated MAP kinases will subsequently activate Hepcidin transcription and consequently its expression (Hancock 2010; Knutson 2010).

Studies by Kawabata et al., (2005) and Nemeth et al., (2005) indicated that TFRs, in particular transferrin receptor 1 (TFR 1) and transferrin receptor 2 (TFR2), are important in regulating the expression of Hepcidin. This observation can be explainable on the basis of the interaction of TFRs, High iron gene (Hfe) and Transferrin, in particular holo transferrin (Knutson 2010). Gao et al's (2009) studies also revealed that iron saturated transferrin (holo transferrin) plays an important role in TFR2 mediated Hepcidin production. The interaction between Hfe and TFR2 upregulates Hepcidin expression, furthermore Hfe is also able to interact with TFR1. TFR1's binding sites for holo transferrin and Hfe overlap (Schmidt et al., 2008).

In their model, Schmidt et al., (2008) supports the notion that Hfe, holo transferrin,

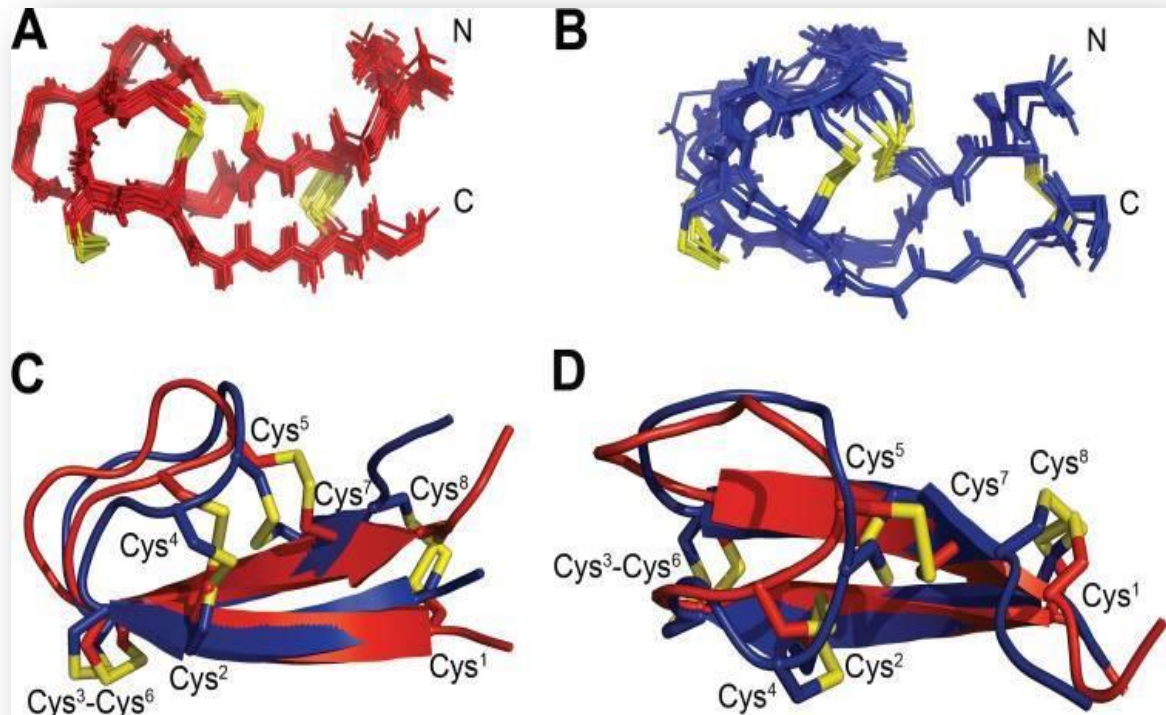
TFR1 and TFR2 form a signalling complex that regulates Hepcidin expression based on the type of interactions at any particular time. In low Iron states where holo transferrin is inadequate most of the Hfe is sequestered in TFR1 preventing its interaction with TFR2 and consequently reducing the signalling for Hepcidin expression. In Iron replete states the levels of holo transferrin are greatly increased. This in turn increases holo transferrin and Hfe competition for the TRF1 binding sites with the resulting increase in available Hfe for TFR2 binding and consequently increased signalling for Hepcidin expression.

Additionally, Hepcidin inhibits TFR expression thereby inhibiting iron transportation. In their work, Du et al., (2011) demonstrated that Hepcidin directly reduces the expression of TFR albeit TFR1 in this case. They concluded that Hepcidin controlled iron uptake and release by regulating the expression of TFRs and other iron transport proteins (Du et al., 2011). It is pertinent to note that the action of Hepcidin on intracellular Iron transportation is two-fold. Through its interaction with TFRs and Divalent Metal Transporters (DMTs), Hepcidin regulates intracellular iron uptake. On the other side it interacts with ferroportin to regulate iron release from the iron rich cells namely macrophages and intestinal epithelial cells.

### **2.6.2. Hepcidin structure**

Hepcidin is a polypeptide which in its mature form can contain up to 25 amino acids (Krause et al., 2000). The mature forms of Hepcidin originate from an 84-amino acid long precursor known as pre-prohepcidin (Clark et al., 2011). This polypeptide, Hepcidin, is folded into an irregular shape held together by four disulphide bonds according to earlier work by Krause et al., (2000). These disulphide bonds link the eight cysteine molecules found in the Hepcidin polypeptide (Krause et al., 2000). The disulphide bonding pattern appear to be constant in all animals capable of producing Hepcidin (Nemeth et al., 2006) and more recent work by Jordan et al., (2009) further elucidated that the Hepcidin molecule forms a hair like structure. The structure exists in two forms depending on the thermal conditions. Figure 2.3 shows the two different structures of Hepcidin as elucidated in Jordan et al's (2009) work. The N terminal end shown below is essential for biological activity and its interaction with ferroportin (Clark et al., 2011)

**Figure 2.3: An illustration of Hepcidin structures at different temperatures.** Reproduced from Jordan J.B., Poppe L., Haniu M., Arvedson T., Syed R., Li V., Kohno H., Kim H., Schnier P.D., Harvey T.S., Miranda L.P., Cheetham J., Sasu B. J. (2009). Hepcidin revisited disulphide connectivity, dynamics, and structure. *J. Biol. Chem.* 284: 24155–24167.



The figure above shows two forms of the three-dimensional structure of Hepcidin A & C at high temperatures and B & D at low temperatures. The four disulphide bonds illustrated above are important for its interaction with ferroportin, to this end it has been demonstrated that cross species Hepcidin ferroportin interactions are possible (Nemeth et al., 2006). This has however posed challenges in assay development as cross species immune recognition of the Hepcidin antigen has a bearing on provocation of immune responses in host animals.

### 2.6.3. Mechanism of Hepcidin action

Despite its antimicrobial action, Hepcidin is known as an iron regulatory hormone and its activity is closely related to its interactions with iron transport proteins. Its role in iron metabolism can be inferred from Pigeon et al's (2001) work where it was observed that its synthesis can be induced by dietary iron.

Hepcidin down regulates ferroportin by regulating its post transcriptional expression

and by degrading expressed ferroportin (Nemeth et al., 2004). These interactions block iron transport from the iron rich cells thereby reducing iron bioavailability. It is pertinent to note that ferroportin is present in cells such as hepatocytes, reticuloendothelial macrophages as well as placental tissue. These are the active sites for the movement of iron and contribute significantly to its bioavailability. The interaction between Hepcidin and ferroportin on the plasma membrane results in the formation of a Hepcidin-ferroportin complex. The interaction is thought to involve biochemical reactions where sulphur anions are exchanged between the disulphide bonds of both Hepcidin and ferroportin (Qiao et al., 2012). This complex is internalised by a process called Hepcidin induced endocytosis. The internalisation results in the reduction in the number of available ferroportin molecules on the plasma membrane surface for iron exportation.

After the process of endocytosis, the Hepcidin-ferroportin complexes are then degraded by tyrosine phosphorylation and ubiquitination of the Hepcidin-ferroportin complex (de Domenico et al., 2007; Qiao et al., 2012). In their work, de Domenico et al., (2007) demonstrated that the attachment of Hepcidin to ferroportin triggers phosphorylation of ferroportin and this takes place at the plasma membrane. Once phosphorylated, the complex is internalised into the cytoplasm where it is dephosphorylated and subsequently ubiquitinated. Ubiquitinated ferroportin is then transported to the lysosomes where it is degraded, and this concludes the fate of both ferroportin and Hepcidin in this interaction.

## **2.7. Hepcidin and iron metabolism**

Iron movement involves its uptake into the cells, in particular apical cells of the intestinal epithelium, and its release into plasma. Iron uptake is usually through absorption of dietary iron from the intestine. Absorption of iron occurs at a smaller scale in comparison to the average daily intake of dietary iron, thus most of the iron just passes through the gastrointestinal tract and is excreted through faecal matter. This can be attributed to the fact that the body does not have a defined physiological mechanism of disposing of iron once it is absorbed. Iron absorption therefore only occurs to replace iron lost due to normal sloughing of cells of the gastrointestinal track, menstrual blood loss and occult blood loss all of which have minimal effect on total available body iron. As a result, only 1 to 2 milligrams of iron needs to be replaced on



a daily basis (Hoffbrand and Moss 2011). The iron requirements are much increased in pregnancy due to the demands attributable to foetal growth and an increase in maternal red cell mass.

Dietary iron occurs as either haem iron or non-haem iron. Meat is the main source of haem iron whereas vegetables and plants are the main sources of non-haem iron. The iron absorption into the cells of the intestinal lumen is facilitated by a transmembrane protein called Divalent Metal Transporter (DMT). Acidic conditions in the intestine help to maintain the solubility of ferric iron ( $\text{Fe}^{3+}$ ). These  $\text{Fe}^{3+}$  molecules are then reduced by ferrous ions ( $\text{Fe}^{2+}$ ) by the action of a ferric reductase enzyme (Kaplan & Ward 2013). Once reduced the ferrous ions are then transported into the cell cytosol by the DMT. Once absorbed into the cells, the iron's release or export from the cells is regulated by another transmembrane protein called ferroportin.

Ferroportin is the only mammalian Iron exporter known so far (Totorsa et al., 2016) thus its role as a major iron transporter makes it solely responsible for regulating iron bioavailability. Hepcidin, in particular Hepcidin 25, acts directly on ferroportin by degrading and internalizing this protein, a relationship which links Hepcidin activity to iron bioavailability. There is a direct relationship between Hepcidin expression and iron levels and the first study to demonstrate this link was performed by Pigeon et al., (2001). Hepcidin levels are reduced in response to low iron stores and this allows more iron to either be released from macrophages or absorbed from the intestines (Collins et al., 2008; Nemeth & Ganz 2009) when iron levels are high there is a consequential increase in the levels of Hepcidin (Collins et al., 2008; Nemeth & Ganz 2009). It is for this reason that Hepcidin plays a role in anaemias associated with iron metabolism.

### **2.7.1. Hepcidin –ferroportin interaction**

Ferroportin is the only exporter of iron in mammals (Donovan et al., 2005; Totorsa et al., 2016), in their review, Kaplan and Ward (2013) also alluded to the fact that it is essential for the distribution of iron between tissues. Ferroportin is responsible for externalization of iron from iron replete cells such as macrophages, hepatocytes and intestinal epithelial cells. On the other hand, Hepcidin is the only known receptor for ferroportin (Poli et al., 2014; Ramey et al., 2010). These attributes for Hepcidin and ferroportin, coupled with the unique relationship between Hepcidin and ferroportin makes Hepcidin the key regulator of iron homeostasis.

In their work, Nemeth et al., (2006) demonstrated that Hepcidin-ferroportin interaction is reliant on the full functionality of the N-terminal end of the hepcidin polypeptide. They also highlighted that disulphide bonds are formed when the interaction is formed (Nemeth et al., 2006). Their later work on disulphide bonds and Hepcidin-ferroportin interaction further elucidated that all four disulphide bonds on the Hepcidin molecule may be involved in the disulphide bond exchange with ferroportin (Clark et al., 2011)

The presence of Hepcidin inhibits the iron export action of ferroportin by destroying it using the mechanisms that have been described earlier in section 2.1.3. This results in the internalization of iron and subsequently reduced extracellular iron.

## **2.8. Hepcidin and Human disease**

Some human diseases can be linked to the way by which Hepcidin is regulated. Knowledge on the behaviour of this hormone is therefore important in trying to elucidate the pathophysiological mechanisms of disorders associated with abnormal iron-Hepcidin interactions. Hepcidin is directly involved in iron interactions and is itself regulated by iron bioavailability as well as hypoxia and erythropoietic activity (Piperno et al., 2009). In normal physiological states, Hepcidin production is up regulated by increased iron stores and inflammatory conditions. This is in an attempt to normalise iron availability. On the other hand, Hepcidin production is down regulated by anaemia and any physiological consequences of anaemia such as reduced tissue oxygen availability and increased erythropoietic activity. This again is in an attempt to maximize iron bioavailability in an effort to correct the anaemic state.

### **2.8.1. Hepcidin deficiency**

Hepcidin deficiency can be due to inadequate production, increased destruction or structural defects within the Hepcidin molecule. Inadequate production can be due to genetic defects within the Hepcidin gene or the regulators of Hepcidin expression. Genetic aberration in HJV, Hfe or TFR expressions can lead to reduced Hepcidin production and ultimately Hepcidin deficiency (Du-Thanh et al., 2011). In such instances ferroportin is overexpressed leading to unregulated increase in dietary iron absorption. In macrophages and intestinal duodenal cells iron is over exported resulting in depleted intracellular iron as well as increased extracellular

iron. The presence of large amount of extracellular plasma iron saturates the iron transporter, transferrin. Once transferrin is saturated it leaves some iron unbound, which finds its way to the liver, heart and other endocrine organs. The liver cells have limited capacity for iron export (Ganz & Nemeth 2012) consequently iron accumulation in the liver is evident before other organs.

Genetic defects within the Hepcidin gene, the HAMP gene, can cause structural changes within the Hepcidin molecule which have an effect on its interaction with ferroportin. In such cases ferroportin degradation and/or internalisation is not effected leading to unregulated iron export from the cells. Unregulated iron export consequently results in systemic iron overload and conditions associated with increased iron bioavailability.

### **2.8.2. Hepcidin excess**

Hepcidin excess could be due to overstimulation of hepcidin synthesis or reduced clearance of normally produced hepcidin. Both scenarios are pathological states or occur as a result of manipulated iron metabolism such as in transfusion iron overload. Blood transfusion results in transitory increase in iron stores. This increase causes excess hepcidin production as a compensatory mechanism to counter the increased iron bioavailability (Nemeth et al., 2006a)

In kidney disease renal clearance of hepcidin and other metabolites is reduced due to decreased kidney function resulting in increased hepcidin levels. Kidney disease results in reduced glomerular filtration and it may also be accompanied by inflammation which in itself is a positive regulator for hepcidin synthesis. It is important to note that hepcidin overexpression as a result of excess hepcidin results in reduced iron bioavailability (Nemeth and Ganz 2009). This will subsequently result in reduced haemoglobin synthesis, a condition known as anaemia of inflammation.

## **2.9. Summary of the aims of the study**

This study aims to assess the clinical usefulness of Hepcidin as a routine test in diagnosing iron deficiency anaemia. The assessment is done by appraising the current diagnostic approaches with Hepcidin assay on participants deemed to be having iron deficiency anaemia. The study also aims to assess the predictive value of Hepcidin in

patients on iron treatment for iron deficiency anaemia. In order to fulfil the above stated aims it is essential to establish baseline Hepcidin values to refer to in a Namibian context. This study therefore aims to establish the reference normal range in an adult population in Namibia. The normal adult population in this context is defined by the NaMBTS donation criteria. Under these criteria, only donors over the age of 16 years are allowed to donate blood (MoHSS, 2010). Participation in research by eligible donors between the age of 16 and 18 years requires parental consent in Namibia. Donors in this age group would present logistical complications to recruit. Donors qualifying to donate blood are regarded as normal healthy adults; a discussion on this criterion is included in the inclusion criteria.

## CHAPTER 3

### 3. RESEARCH METHODOLOGY AND DESIGN

#### 3.1. Introduction

The aim of this research was to investigate how Hepcidin testing can help to improve the diagnosis of Iron Deficiency Anaemia (IDA) and ultimately improve its management. This has been prompted by the high prevalence of this ailment in developing countries. In order to fulfil this aim, specific objectives including establishment of reference normal values for Hepcidin and characterisation of the quantitative behaviour of Hepcidin in iron deficiency anaemia were set.

Establishment of reference normal values required the use of blood samples from healthy normal adults. In contrast, an investigation on the quantitative behaviour of Hepcidin in anaemia required blood samples from individuals with iron deficiency. The laboratory experiments for full blood count, Hepcidin and serum iron were however the same. The research design and experimental methods to address the two objectives were similar in many ways. There were however some unique features in the populations under study thus the designs are discussed in separate sections.

#### 3.2. Establishing reference normal values for the Namibian population

Hepcidin testing is not currently being performed in routine diagnostic laboratories. Introducing this biomarker into the diagnostic arena requires the establishment of reference normal ranges adjusted for age and gender. Pasricha et al., (2011) established reference normal ranges for Hepcidin as <5.4 ng/mL to 174.6ng/mL in healthy premenopausal women in Australia. Galesloot et al., (2011) also established normal ranges for Hepcidin in the Netherlands. The later were divided according to gender and age in strata of 5 years each. The more recent work on Hepcidin reference normal ranges was performed in Greece by Sdogou et al., (2015) and it provided useful information relevant to the Greek population albeit for the paediatric populace. Appropriate reference normal ranges are required for accurate diagnosis and prognosis of disease, however, for Hepcidin most of this work has been performed

outside Africa South of Sahara. It is therefore justifiable to carry out the same work in an African context given the differing geographical locations and ethnicity compositions.

The establishment of reference normal ranges in Namibia form the baseline and reference point for studying quantitative characteristics of Hepcidin. Establishing these ranges in Namibia also gives an opportunity to compare them with already established data on normal ranges elsewhere. The other importance of locally reputable reference normal ranges is the possibility of establishing Hepcidin-Hb correlations of which the data can also be compared to the same correlations found elsewhere. Sampling for normal healthy adults was done among the blood donor population.

### **3.2.1. Sampling and sample size calculations for determination of reference normal values.**

Normal healthy blood donors were used to establish reference normal ranges. Healthy individuals were defined according to the donation criteria as set out by the NaMBTS. The donation criteria states that individuals should be between the age of 16 and 65 and in good health for them to be able to donate blood (MoHSS 2010). It also states that they should have the following attributes:

- No recent alcohol intake to an extent that someone is deemed legally unfit to drive or operate machinery.
- Prospective donors should weigh more than 50kg. This attribute protects donors from developing anaemia of transfusion.
- There should be no clinical evidence of bacterial infection on presentation. Blood transfusion carries a risk of bacterial contamination. Excluding prospective donors with such infections therefore reduces the risk of bacterial contamination.
- Leading a sexually safe life style by practicing safe sex Safe sex practices includes the use of protection and having one partner at a time.
- Are committed to helping others and will therefore donate for the right reasons (MoHSS 2010).

The profile of a blood donor in general is consistent with a healthy individual and it

suffices to say blood donors are a good representative of a healthy population.

The initiative by the NaMBTS to recruit Voluntary Non-Remunerated Donors (VNRD) makes it possible to use this population to establish reference normal ranges. These VNRDs are conscious of their contribution to society and they generally have good healthy habits making them a better target than randomly selecting individuals outside of a healthcare establishment. VNRDs give blood freely and voluntarily without expecting anything in return (WHO, 2012). Research by Kalargirou et al., (2014) established that the majority of these donors donate blood out of their unselfish concern for the welfare of others. These attributes are good features that contribute to the general wellbeing of an individual. They also demonstrate the appreciation of health for both self and others.

In their work, Sdogou et al., (2015) carried out physical examinations on their participants to help ascertain their health status. In this research, the NaMBTS procedures as stated in their guidelines on donor selection (MoHSS 2010) were used. The procedures include checking for vital signs such as blood pressure, weight as well as asking questions about the donors' general health using a standard donor questionnaire. The donor questionnaire elicits for information on an individual's health and medical history of which some certain criteria has to be met before being allowed to donate blood.

The approach on using donor questionnaires is similar to what Galesloot et al's (2011) research used where they employed a health and lifestyle questionnaire as one of their participant selection tools. These approaches ensure that the participants' health status is assessed based on clinical presentation on the day of blood sample collection as well as relevant medical history. This holistic approach minimises confounding variables such as hidden medical conditions that interfere with the physiological mechanisms involved in Hepcidin metabolism. Given that Hepcidin is sensitive to certain medical conditions such as bacterial infections, it is important to exclude them.

On that premise individuals who qualified to donate blood were therefore included in the study. Deferral, whether permanent or temporary, was one of the exclusion criteria the basis of which was its perceived deviation from how "normal healthy" adult is defined. Non-consenting participants were also excluded as it is ethically immoral to

coerce anyone to participate in research studies. The other inclusion/exclusion criteria are tabulated in Table 3.1.

**Table 3.1: The Inclusion and exclusion criteria for participants enrolled in determining the normal range for Hepcidin in the Namibian population.**

<b><i>Criterion</i></b>	<b>Samples for group 1. Normal control samples</b>
<b><i>Inclusion:</i></b>	<ul style="list-style-type: none"> <li>• Samples from healthy donors who are allowed to donate at the time of joining the study.</li> <li>• Samples from apheresis donors donating platelets.</li> <li>• Samples from stable regular blood donors attending the blood donation sessions for the purpose of donating blood.</li> <li>• Samples with normal serum iron results.</li> </ul>
<b><i>Exclusion:</i></b>	<ul style="list-style-type: none"> <li>• Samples from donors who have features suggestive of Iron deficiency anaemia such as a low haemoglobin and low serum iron levels.</li> <li>• Samples from non- consenting participants.</li> <li>• Samples collected from individuals not donating blood.</li> <li>• Samples from participants who are therapeutic donors.</li> </ul>

The inclusion and exclusion criteria for this study were heavily informed by the MoHSS guidelines and were able to exclude donors with conditions that might interfere with the establishment of reference normal values for Hepcidin. These included donors with low haemoglobin levels as well as those with recent bacterial infections. Bacterial infections are known to upregulate Hepcidin expression and consequently reduce iron bioavailability (Armitage et al., 2011). Such participants will invariably exhibit higher Hepcidin levels. Excluding donors with recent infection is therefore mandatory for reference normal range values to be a true reflection of the normal healthy population.

After establishing the inclusion criteria, a sample size calculation was performed using a sample size calculation for quantitative variables obtainable from cross sectional studies. The formular for sample size calculation is shown below and has been described in Charan and Biswas (2013).



$$\text{Sample size} = \frac{(Z_{1-\alpha/2})^2 SD^2}{d^2}$$

Where:  $Z_{1-\alpha/2}$  = standard normal variate

SD= standard deviation. The SD value is obtainable from previous work.

d= absolute error or precision.

The standard normal variate used in this study is 1.96 using 5% type 1 error ( $P < 0.05$ ) and the desired absolute error/precision (d) of 6ng/ml. The desired absolute error was selected taking into consideration the performance characteristics of the CUSABIO® (n.d.) ELISA kit. The kit has a detection range of 4.69ng/mL-300ng/mL and a minimum detectable dose of less than 1.17ng/mL.

The standard deviation was obtained after examining the literature with similar studies such as the work of Sdogou et al., (2015), Pasricha et al., (2011) and Galesloot et al., (2011). An SD of 18.94ng/ml was used.

$$\begin{aligned} \text{Sample size} &= \frac{(1.96)^2 \times (18.94)^2}{(6)^2} \\ &= 38.28 \end{aligned}$$

A sample size of 40 was therefore used to establish the normal range for Hepcidin using the blood donor population without partitioning for age and gender.

### 3.2.2. Ethical issues

Before embarking on this project approval was sought and obtained from the University of Bath through the Research Ethics Approval Committee for Health (REACH). The REACH reference numbers pertaining to this work are EP 14/15 237 – for when the project was initially reviewed and EP 15/16 20 – for when the project was approved. In Namibia permission to carry out research work and ethical clearance was also sort and granted from the Namibian Blood Transfusion Service before any work could be carried out. A copy of the approval letter is attached as Appendix 2. The Namibia Blood Transfusion Service has a mandate from the Ministry of Health and Social Services in Namibia to carry out research on blood transfusion practice and blood transfusion related work. This research looked at anaemia which has a direct link to

the activities of the NaMBTS as an establishment. The participants were also recruited from the NaMBTS therefore the application for ethical approval had to be directed to the NaMBTS. Another application to conduct research in Namibia was also sought from The Ministry of Health and Social Services. Permission was granted and copy of the approval letter from the MoHSS is attached as Appendix 3.

Written informed consent was obtained from all blood donor participants and samples were collected into tubes containing Ethylene Diamine Tetra-acetic Acid (EDTA) as an anticoagulant and another set into serum separator tubes (SST) which do not contain an anticoagulant. Samples in the EDTA tubes were used to perform a full blood count and those in SST were used to assay for Hepcidin and serum iron levels. The blood donors were presented with a participant information sheet during the donation sessions. Both the participant information sheets and consent forms were presented in two languages namely English and Afrikaans. The participant information sheets (copies attached as Appendices 4 and 5) provided information on the relationship between Hepcidin and Iron. It also had useful information on iron deficiency anaemia and all the information was written in a language that a layperson would understand. The prospective participants were given the opportunity to read and ask questions on the content of the information sheet. Questions raised from the information sheet were answered by the nursing staff in the first instance and only escalated to the primary researcher when the nursing staff could not address them fully.

Once the prospective participants were satisfied, they were given consent forms to complete before blood samples were taken. The consent forms documented information such as the participant identification number and the numbers used were the donation numbers which corresponded with the sample number. Both the participant consenting to take part and the person taking consent were required to sign after going through the consent form. The English and Afrikaans version copies of the consent forms are attached as Appendices 6 and 7 respectively. The patient information sheets as well as the consent form were provided in Afrikaans to cater for participants who were more comfortable with this language. Namibia uses English as the official language but has a significant size of native Afrikaans speaking individuals.

Any adverse effects of blood collection were dealt with in the same manner as adverse effects for blood collection procedures of which are part of the NaMBTS standard operating procedures. The NaMBTS is an establishment which deals primarily with

blood collection, processing and distribution thus the nursing staff are conversant with procedures to follow when there are any signs of untoward effects of blood collection. This staff group was the same group that assisted in blood sample collection for this study.

### **3.2.3. Data collection for use in determining the reference normal values**

#### **3.2.3.1. Sample collection**

Venous blood samples were collected from consenting healthy blood donors by the NaMBTS nursing staff. Before blood collection, participants' weight and vital signs such as blood pressure were determined to ensure they were safe to give blood. Normal phlebotomy procedures were followed as described in the WHO guidelines on phlebotomy procedures (WHO 2010). The participants were made to sit in a chair in a quiet and comfortable environment and a tourniquet was applied on the arm. The tourniquet makes it easy for the phlebotomist to identify the vein to use for blood collection. Once the vein was identified the venepuncture site was disinfected with an alcohol based disinfectant and allowed to dry before inserting the blood collection needle. The blood collection needle was inserted, and blood collected into vacutainer tubes containing EDTA as an anticoagulant and another sample into an SST tube. Two samples in total were collected from each consenting participant and were used to perform full blood count, Hepcidin and serum iron assays.

The participants then proceeded to donate blood after samples for the Hepcidin study were collected. This order of events was important in order to avoid variation in Hepcidin concentrations caused by changes in blood volumes post blood donation. Hepcidin values respond to decrease in blood volume because of the consequent reduction in iron content (Collins et al., 2008). The samples were also collected from donors presenting at the NaMBTS for donation in the morning in order to minimise the effects of diurnal variation in Hepcidin (Kroot et al., 2009). The timing of collections was also important in optimising sampling conditions and it also became pertinent to standardise preparation of samples for laboratory analysis.

#### **3.2.3.2. Measures to ensure sample validity**

Two sets of samples were collected at a time, one for the Full Blood Count (FBC) test and the other sample for the Hepcidin and serum iron assay and any other assays

related to this study. The samples for FBC were collected in an EDTA anticoagulant which prevents clotting while preserving the morphology of the cells. The samples used for Hepcidin and serum iron were collected in a serum separator tube (SST) which does not have any anticoagulant. The samples for FBC test were taken to the laboratory where they were processed on the same day. Samples for FBC need to be tested as soon as possible because storage, regardless of conditions, affects the different parameters of FBC results. This is in line with the recommendations and advice from different authors who have done some work on FBC results and storage conditions (Turhan et al., 2011; Cornet et al., 2012).

The second set of samples that were collected in SST tubes were kept in the refrigerator at 2-8°C at the Namibia Blood Transfusion Service awaiting collection and transportation to the laboratory for processing. In the laboratory the samples were centrifuged at 1000g for 15 minutes according to the kit manufacturer's instructions (CUSABIO® n.d.). The samples were then aliquoted and frozen at -70°C to avoid loss of bioactivity of the Hepcidin molecules. All samples were stored for at most 2 months before testing in line with the guidance from the Hepcidin kit manufacturer (CUSABIO® n.d.) ELISA Kit). Storing samples for longer than 2 months is not recommended as it can result in the degradation and denaturalisation of the Hepcidin protein. The storage conditions were monitored closely both at the NaMBTS as well as in the testing laboratory. At NaMBTS the fridges are monitored to ensure records are generated to support the cold chain initiative as dictated by the NaMBTS establishment. At the testing laboratory the freezer temperatures were monitored as part of the routine monitoring exercise, records of which are stored and archived as per standard operating procedures in place at the Namibia University of Science and Technology (Blaauw, 2016).

On the days of the experiments samples were thawed and allowed to reach room temperature before being assayed. All the other reagents were also brought to room temperature as per the manufacturer's guidance. Once thawed the samples were spun down at 1000g for 5 minutes. All reagents were prepared according to the assay procedure (CUSABIO® n.d.) and an ELISA was set up.

Samples for the Hepcidin assays were also used for assessing serum iron levels. Serum iron results were obtained to confirm the presence of normal iron levels in this

group. On the day of analysis samples were thawed and working reagents were prepared immediately prior to use to ensure the validity of the assay is not compromised. The thawed serum samples were spun at 1000g for 5 minutes to remove any insoluble particles that might interfere with spectrophotometric characteristics of the coloured complex when reading the absorbance of the final solution. According to the kit insert, samples needed to be separated as soon as possible. This was consistent with the requirements of the Hepcidin assay protocol therefore the same sample preparation protocol was sufficient to ensure sample validity.

Assessment of sample integrity was paramount and factors such as haemolysis were used to exclude the sample for analysis. Haemolysis results in release of iron from red blood cells and subsequent increase in serum iron levels (Hoffbrand and Moss 2008). Samples for serum iron as well as Hepcidin assays were only collected in the morning to minimise the effect of diurnal variation on these two analytes.

Reagents for the serum iron assay were protected from light and left over prepared solutions were kept for not more than 3 months in the refrigerator at 2-8°C according to the reagent manufacturer's instructions set out in the serum iron kit insets. A copy of the kit insert is included as Appendix 1.

#### 3.2.3.3. Hepcidin and serum iron assays

The Hepcidin assays were run using an ELISA technique with a detection range of 4.69ng/ml-300ng/ml. The inter assay and intra-assay precision for this assay was <10% and <8% respectively (CUSABIO® n.d.). The intra-assay Precision was performed by the kit manufacturer. This was carried out using three samples of known Hepcidin concentration. The samples were tested twenty times on one ELISA plate and the results used to assess intra assay precision (CUSABIO® n.d.). The inter-assay Precision to determine Precision between assays was also performed by CUSABIO® who are the kit manufacturers. On this instance three samples with a known concentration of Hepcidin were tested in twenty assays and the results used to assess the inter-assay precision. Precision tests were not run on this study due to resource limitations.

#### 3.2.3.4. Hepcidin ELISA assay

The principle of the assay used is based on the quantitative sandwich enzyme immunoassay technique as described in the Hepcidin kit insert (CUSABIO® n.d.) ELISA Kit). The principle is as follows: Microtiter plates with wells that are pre-coated with anti-hepcidin 25 antibodies are used. The pre-coated antibodies in the wells specifically react with hepcidin 25 antigens in the sample to form an antigen- antibody complex. The reaction is washed to remove any unbound material leaving the complexes attached to the surface of the microtiter plate wells.

After removing any unbound material, an anti hepcidin 25 antibody conjugated to biotin is introduced. The conjugated anti Hepcidin antibody react with the available antigenic determinants on the hepcidin 25 molecule. The mixture is washed to remove any unbound material before avidin conjugated Horse Radish Peroxidase is introduced into the wells of the microtiter plate. Addition of an enzyme conjugated to avidin allows for the binding of biotin with avid to form a stable interaction. The final complex has the ability to act on a substrate because of the presence of the enzyme Horse Radish Peroxidase (HRP).

The peroxidase enzyme acts on the substrate to produce a coloured product. The reaction takes place at 37°C for 15-30minutes. In this assay the time was set at 30 minutes in order to standardise the protocols. This duration was chosen as the standard incubation time as it allowed maximum time for the reaction to complete. After the incubation period, a stop solution is added to stop the reaction and intensity of the colour formed is measured at 450nm. According to Beer Lambert's law, the intensity of the colour so formed is proportional to the concentration of the substrate, in this context the concentration of hepcidin 25 in the blood sample. This was obtained from a standard curve which was constructed using Hepcidin standards supplied by the kit manufacturer.

There are several methods to quantitate hepcidin which include the ELISA, Mass Spectrometry (MS), High Performance Liquid Chromatography (HPLC), Radio Immunoassays (RIA), and other novel approaches such as Surface-Enhanced Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (SELDI-TOF MS) and Liquid

Chromatography Tandem Mass Spectrometry (LC/MS-MS). In their review, Macdougall et al., (2010) discussed the different methodologies available for Hepcidin testing including the ELISA technique. In the same review a number of studies done on Hepcidin using the ELISA technique were discussed. They noted that Koliaraki and colleagues as well as Ganz and colleagues' work on ELISA conferred that there is no cross reactivity between the hepcidin isoforms when using the ELISA technique Koliaraki et al., 2008 in Macdougall et al., (2010); Ganz et al., 2008 in Macdougall et al., (2010). It is also important to highlight that the Hepcidin kits used for this study noted that there is no significant cross reactivity between hepcidin25 and the other isoforms (Cusiobio® n.d.). This feature is important because the work on this research aimed at characterising the dynamics of hepcidin 25, excluding other isomers, in relation to other variables as described.

An ELISA based immunoassay was chosen because its performance is comparable to other technologies. It is also relatively cheap in comparison to technologies such as Radioimmunoassay (RIA) and mass spectrometry (MS). These two attributes make an ELISA the right choice for an assay in the setting in which Hepcidin testing is being proposed. The proposed setting is the testing of Hepcidin in patients with IDA in a developing country where health economies are already scanty and stretched. A simple ELISA based assay provides a solution as it does not require huge capital investments on equipment for the implementation of this assay. The ELISA only requires a reader and an incubator as opposed to mass spectrometry which requires a mass spectrometer that is more expensive than a reader and incubator combined

#### 3.2.3.5. Serum iron colorimetric assay.

The serum iron assay used in the experiments is based on the measurement of the intensity of the colour that forms when ferrous ions ( $\text{Fe}^{2+}$ ) react with FerroZine. The assay is sometimes called the Ferrozine method, deriving its name from the ferroin compounds that are used. Ferrozine is a chemical compound that forms a stable coloured compound when it is complexed with ferrous ions ( $\text{Fe}^{2+}$ ) (PubChem 2016). The complex so formed has an absorption peak at 562nm thus the absorbances for serum iron determination using this method were carried out at 562nm. The ferrous ions are liberated from iron complexes and subsequently reduced

from ferric ( $\text{Fe}^{3+}$ ) to ferrous state ( $\text{Fe}^{2+}$ ) by a weak acid (CHRONOLAB n.d.) In this assay ascorbic acid was used as the weak acid and this was supplied as reagent 2 (R2) in the kit. The information on the other reagents contained in the iron kit is shown in the kit insert in Appendix 1.

The reaction between ferrous ions and FerroZine results in the formation of a coloured complex. The intensity of the colour so formed is proportional to the concentration of iron in the sample. The colour intensity was measured by a spectrophotometer at a wavelength on 562 nanometres (nm). By using a standard with known concentrations of iron the beer lambert's law could be applied to calculate the concentration of iron in the participants' samples. This assay was performed manually and required the use of a spectrophotometer.

### **3.2.4. Laboratory methods**

Full blood count, serum iron and Hepcidin assays were performed on the blood samples that were collected in EDTA and SST tubes respectively. All three tests were performed in the laboratory in Namibia. Tests for full blood count were performed on the day of sample collection while assays for Hepcidin and serum iron were performed at a later date. Samples for the latter were kept frozen until the assays were ready to be put up.

#### **3.2.4.1. Full blood Count Analysis.**

The full blood count tests were performed using a Pentra XL 80 full blood count analyser and were analysed on the day of sample collection. The Pentra XL80 is a 5-part differential analyser which can perform a full blood count and differentiate white blood cells. The white blood cells can be differentiated into neutrophils, lymphocytes, monocytes, basophils, eosinophils. The white cell differential can be extended to include atypical lymphocytes (ALY) and large immature cells (LIC) but these parameters are currently for research use only. This analyser uses electrical impedance as a principle for the detection and quantitation of red blood cells and spectrophotometry for determination of haemoglobin concentration (ABX user manual n.d.).



The principle of electrical impedance is based on the concept of differences in electrical resistivity between different cellular components of blood as well as plasma which is the acellular element of anticoagulated blood. The architecture or morphological differences between the cells determine the level of resistivity and these differences can be used to differentiate the types of cells. A sample of blood is mixed with a diluent solution which conducts electricity and the mixture is passed through an aperture with an electrical current. The aperture diameter for the ABX XL80 analyser is 50µm large (ABX user manual n.d.). As the blood mixture passes through the aperture, cells offer more resistance than plasma and they generate peaks which are then enumerated to give the number of cells. These are then computed by the analyser and expressed as number of red blood cells/µL.

The principle used for haemoglobin, spectrophotometry, is different from that for red blood cell counting. Spectrophotometry for Hb determination is based on the measurement of absorbance of RBC ABX lyse mixture (ABX Technical manual 2002). The lyse reagent contains potassium cyanide at 0.03%, ammonium salt and phosphate buffered saline (ABX Technical manual 2002). The lyse reagent lyses red blood cells and they release haemoglobin which then combines with the potassium cyanide to form cyan methaemoglobin. The absorbance of the cyan methaemoglobin compound is then measured at a wavelength of 550nm (ABX Technical manual 2002). The absorbance is converted using beer lambert's law which states that the absorbance of a compound is proportional to its concentration (Swinehart 1962).

#### 3.2.4.2. Serum iron assay

The serum iron assay was performed on all samples from participants meeting the inclusion criteria for normal adults as defined by the NaMBTS donation protocol. The serum iron levels were measured using a colorimetric assay the principle of which has been described in section 3.2.3.4 above. A working reagent was prepared by dissolving ascorbic acid in an acetate buffer of pH 4.9 according to the manufacturer's instructions which are attached as Appendix 1. The solution was mixed until all the contents were dissolved, and this was labelled as the working reagent (WR). 1000µL of the working reagent was pipetted into all the cuvettes followed by 3 drops of the FerroZine reagent. The FerroZine reagent was placed in all cuvettes except the sample blank cuvette. Before incubating at 37°C for 5 minutes, 200µL of

the standard, distilled water and samples were put into the standard, blank and samples cuvettes respectively.

The solutions were incubated for 5 minutes and the optical densities (OD) of the mixtures were measured using a spectrophotometer. This was done at 562nm. An absorbance of the blank solution was also taken at the same wavelength of 562nm. This was subtracted from the sample absorbance and the concentration of iron was calculated as follows:

Iron ( $\mu\text{g/dL}$ ) =  $\frac{\text{OD Sample} - \text{OD blank}}{\text{OD Standard}} \times 100$  (concentration of the standard).  
The results were expressed in  $\mu\text{g/dL}$  but a conversion factor of 0.179 could be used to convert the units for serum iron from  $\mu\text{g/dL}$  to  $\mu\text{mol/L}$ .

#### 3.2.4.3. Hepcidin assay

The samples for Hepcidin assaying were tested in duplicate using the Hepcidin ELISA kits supplied by CUSIBIO®. Anti Hepcidin antibody pre-coated wells were supplied in the kit. The wells came in strips with twelve wells on each strip. One hundred microlitres (100 $\mu\text{l}$ ) of prepared standards and samples were added into the wells and incubated for 2 hours at 37°C. At the end of the incubation period the liquid contents of the wells were decanted. 100 $\mu\text{l}$  of biotin labelled antibody was added to each well and the mixture incubated for 1 hour at 37°C. After which the liquid from each well was removed by aspiration. The wells were washed 3 times using the wash solution provided by the kit manufacturer. Subsequently, 100 $\mu\text{l}$  of avidin conjugated to horse radish peroxidase (HRP) was added to each well and incubates for 1 hour at 37°C. At the end of the incubation period the HRP-avidin mixture was aspirated before the wells were washed 5 times with the wash solution. 90 $\mu\text{l}$  of the Tetramethylbenzidine (TMB), a peroxidase substrate was added in the wells and incubated for 30minutes at 37°C.

The plates were protected from light to avoid it interfering with the colour development. The colour reaction was stopped by the addition of 50 $\mu\text{l}$  of stop solution before reading the optical densities for each well at 450nm. The optical density was read using the Biotek ELx800 microtiter plate reader, an automated plate reader from BioTek®. In

each plate all the seven standards and a blank were present in duplicate. The standards had the following Hepcidin concentrations according to the kit manufacturer's instructions: 300ng/ml; 150ng/ml; 75ng/ml; 37.5ng/ml; 18.75ng/ml; 9.375ng/ml; 4.6875ng/ml. The logarithmic values of these concentrations were plotted against the logarithms of the optical densities obtained and this was the standard curve used to calculate sample results. The initial results obtained from the standard curve were logarithms of the sample hepcidin concentrations. The anti-logarithms (anti-log) were then determined to come up with the actual hepcidin concentrations.

### **3.3 Quantitative behaviour of Hepcidin in IDA pre- and post- iron treatment**

Assessment of Hepcidin values in anaemia helps to elucidate the utility of this analyte in the diagnosis and prognosis of iron deficiency anaemia. In this study samples of participants with iron deficiency anaemia were used to assess Hepcidin values and its characteristics. This was done by measuring the values of Hepcidin, serum iron and full blood count parameters such as haemoglobin. The same participants were then followed up for subsequent samples to assess the effect of iron administration on serum iron and Hepcidin levels as well as full blood count parameters. Results from the reference normal range group were also combined with work from the iron deficiency anaemia group in order to fully investigate the quantitative behaviour of Hepcidin.

Statistical models such as multiple regression and correlation analysis were used to assess the value of Hepcidin in comparison to other parameters such as serum iron. This was performed on data obtained from both normal healthy participants as well as iron deficient group. The inclusion of data obtained from normal healthy adults as well as iron deficient participants makes it possible to assess how well Hepcidin is able to differentiate between health and disease states.

Hepcidin values are generally reduced in iron deficient states (Young 2009) to allow for compensatory increase in iron bioavailability. As already elucidated by Nemeth et al., (2004), hepcidin directly controls iron regulation by influencing the activity of ferroportin, the only known iron intracellular exporter. Given its direct role

in iron regulation, Hepcidin could therefore be used as a diagnostic indicator or even more so a prognostic indicator for disorders associated with iron metabolism. Hepcidin values are reduced in iron deplete states in order to facilitate iron loading. A reduction in Hepcidin allows for increased availability of ferroportin, an important player in transmembrane iron transport. Indeed Pasricha et al., (2014) has demonstrated that depressed hepcidin levels are a good indicator of increased iron uptake. The reduction in hepcidin values can however be masked by the presence of other inflammatory conditions such as infections and malignancies. This drawback is not unique to hepcidin alone, current markers of iron dysregulation such as ferritin levels are also affected by inflammation (Hoffbrand and Moss 2011). This drawback should therefore not be discouraging in the quest for better diagnostic strategies for iron deficiency anaemia.

### **3.3.1. Characteristics of Hepcidin in relation to haemoglobin values**

Hepcidin expression is decreased in anaemia and any other conditions which promote increased erythropoietic activity (Nicolas et al., 2002; Pak et al., 2006). Anaemia signals for increased erythropoietic activity as a compensatory mechanism for the reduced haemoglobin levels. Intravenous or subcutaneous administration of erythropoietin also increases erythropoietic activity in an effort to increase the haemoglobin levels. In both cases more iron is needed for haemoglobin synthesis hence increased iron bioavailability is indicated. Hepcidin is therefore suppressed in response to the need for more iron; in fact, Mazur et al's (2003) studies demonstrated that iron levels regulate hepcidin expression albeit in a mouse model.

Anaemia results in decreased tissue oxygenation and increased erythropoietin production. This is due to the effect of hypoxia or reduced renal tissue oxygenation which enhances erythropoietin gene expression. This will subsequently result in increased erythropoiesis with consequent reduction of serum and tissue iron (Pak et al., 2006). This myriad of events has a combined and an individual inhibitory effect on Hepcidin expression. Nicolas et al., (2002) alluded to the fact that decreased tissue oxygenation on its own can suppress Hepcidin expression.

The reduction in haemoglobin results in reduced Hepcidin levels and inversely an increase in haemoglobin levels upregulates hepcidin expression. These characteristics make Hepcidin a potentially good biomarker for conditions where the physiological

balance of iron is defective such as in iron deficiency anaemia.

### **3.3.2. Sampling and sample size calculations in the iron deficiency anaemia participants**

Donors who were temporarily deferred from donating blood due to low haemoglobin levels were recruited to participate in this study. These were otherwise healthy individuals in good health apart from the presentation of anaemia. As alluded to in our previous research, anaemia is a major cause of deferral among blood donors (Gonzo et al., 2016). Given the geographical prevalence data, most anaemia cases in Namibia are due to iron deficiency (WHO 2015). It therefore became clear that the blood donor population makes for a good population to study Hepcidin characteristics in iron deficiency anaemia.

A pre-test/post-test study of Hepcidin, full blood count and serum iron parameters was carried out using a cross sectional study design. The design was chosen as it enabled the researcher to assess the quantitative behaviour of Hepcidin and other related parameters pre- and post-iron treatment. This design may be considered as a variation of standard cross-sectional studies because it involved two sets of cross-sectional data collection on the same sample.

A sample size of 40 participants fulfilling the inclusion criteria was used based on the same calculation as for the Hepcidin normal reference range group given that similar study designs were used in both occasions. The sample size calculation is discussed in earlier in section 3.2.1. and in our article (Gonzo et al., 2017). The same sample size was recruited for the three groups throughout this research work. The three groups were; Hepcidin reference normal range group, pre-iron therapy iron deficiency anaemia group and the post iron therapy iron deficiency anaemia group. Samples from the Hepcidin reference normal range group were used in laboratory experiments carried out in chapter 5 to establish baseline Hepcidin levels in normal healthy individuals. Participants in the iron deficiency anaemia group were followed up after iron therapy and the same sample size was maintained. These participants were randomly selected from participants fulfilling the inclusion criteria. In total 240 blood samples were collected, two from each participant. The two samples were sample with an EDTA anticoagulant for full blood count test and an SST sample without an anticoagulant for Hepcidin and serum iron assays.

Data on Hepcidin, serum iron and full blood count results was collected from donors who were temporarily deferred because of low haemoglobin levels due to iron deficiency. Low haemoglobin was defined as haemoglobin level below 125g/L according to the NaMBTS guidelines (MoHSS 2010). Their condition, iron deficiency anaemia, was assessed by performing a full blood count and serum iron levels. Full blood count results can confirm the presence of anaemia and this was one of the inclusion criteria for this participant group. Another inclusion criterion was the presence of a low serum iron value in participants with low haemoglobin results. This combination of results was used to define iron deficiency anaemia. The temporarily deferred donors that were recruited into this study met all the donation criteria except the low haemoglobin. This made them good candidates for assessment of hepcidin in iron deficiency anaemia because other interfering conditions such as bacterial infection were excluded by the blood donation criteria. This criterion has been discussed in detail in section 3.2.1. The other inclusion/exclusion criteria for the iron deficiency anaemia group are tabulated in Table 3.2.

**Table 3.2: The inclusion and exclusion criteria for participants enrolled in establishing the quantitative behaviour of Hepcidin in the iron deficiency participant group.**

	<b>Samples for group 2: Iron deficiency anaemia samples</b>
<b>Inclusion criteria:</b>	<ul style="list-style-type: none"> <li>• Samples from donors failing CuSO<sub>4</sub> test and have low serum iron.</li> <li>• Samples from temporarily deferred donors with features suggestive of iron deficiency anaemia.</li> <li>• Samples from donors who qualify to donate blood except for the low Hb. These donors were temporarily deferred and asked to come back later.</li> <li>• Samples from blood donors agreeing to give a second set of blood samples on their follow-up visit with the NaMBTS.</li> </ul>
<b>Exclusion criteria:</b>	<ul style="list-style-type: none"> <li>• Samples from donors deferred for reasons other than low Hb.</li> <li>• Samples from therapeutic donors</li> <li>• Samples from participants who fail to come back for testing after taking iron treatment.</li> </ul>

The same donors were followed up for a subsequent sample after administration of iron to assess the effect of iron supplementation on hepcidin values. The same samples were also used to confirm that the intervention of iron has corrected the anaemia, a situation which further confirms the presence of iron deficiency anaemia in the initial samples.

### **3.3.3. Data collection and ethical approval**

Venous blood samples were collected from consenting temporarily deferred donors who had failed the haemoglobin test. The haemoglobin test was performed at the donation site by the NaMBTS staff. The haemoglobin test performed at NaMBTS was a copper sulphate specific gravity test. The principle of the copper sulphate test is based on the ability of a drop of free-flowing blood to sink in a copper sulphate solution with a specific gravity of 1.053g (Mohan et al., 2016). Blood sample with haemoglobin levels above 125g/L would sink and samples with lower haemoglobins would fail to sink. The blood was collected from a finger prick using a capillary tube. After each copper sulphate haemoglobin test the participants were classified as passing (normal haemoglobin) or failing (low haemoglobin) the copper sulphate test based on the behaviour of the blood sample in copper sulphate solution.

The participants who failed this initial  $\text{CuSO}_4$  screening test were deemed to have low haemoglobin. The low haemoglobin results were then confirmed by a full blood count test in the laboratory. The full blood count gave a haemoglobin value as well as more useful information such as the red cell indices. On the day of initial sample collection, the participants were informed of the need to collect a subsequent sample after they have undergone iron treatment. Participants consenting to providing the two samples were then enrolled into the study. These participants were also informed of their option to withdraw consent at any stage should they not wish to continue.

The temporarily deferred donors were counselled by the NaMBTS staff regarding their haemoglobin status before they were invited to participate in the study. Two samples were collected from each participant, one in EDTA and another in an SST tube. The sample collected in EDTA was used to perform a full blood count and the clotted sample collected in an SST tube was used for the serum iron and Hepcidin assays. The subsequent samples were collected three months after the initial sample

and the sample types were identical to the initial ones. Samples were collected from prospective blood donors presenting at the blood donation clinic any time before midday. The timing of blood collection was necessary to minimise the effect of diurnal variation on hepcidin and serum iron levels. This practice was also adopted to ensure consistency with blood sample collection procedures performed on the reference normal range group. The same tests and analysis were performed for both groups therefore the same measures to ensure sample validity were employed.

The application for ethical approval for this study covered both the establishment of reference normal ranges for hepcidin reported earlier in the previous chapter and the characterisation of Hepcidin in iron deficiency anaemia. The ethical issues were identical for both groups; apart from the need to collect subsequent samples in the iron deficiency group, therefore the permissions that were sought covered the whole study. The permissions were sought and granted from the University of Bath REACH, Namibia Blood Transfusion Service and the Ministry of Health and Social Services in Namibia.

#### **3.3.4. Laboratory methods**

##### **3.3.4.1. Hepcidin assay**

The assay for Hepcidin testing was the same for all three experiments in order to maintain consistency. The Hepcidin ELISA test from Cusabio was used to assess hepcidin 25 (Hepcidin) levels the principle of which is described earlier in the previous chapter. On this occasion samples for hepcidin analysis were separated and frozen on the day of collection. These samples were kept frozen at -70°C for not more than 2 months as per kit manufacturer's recommendations (CUSABIO® n.d.).

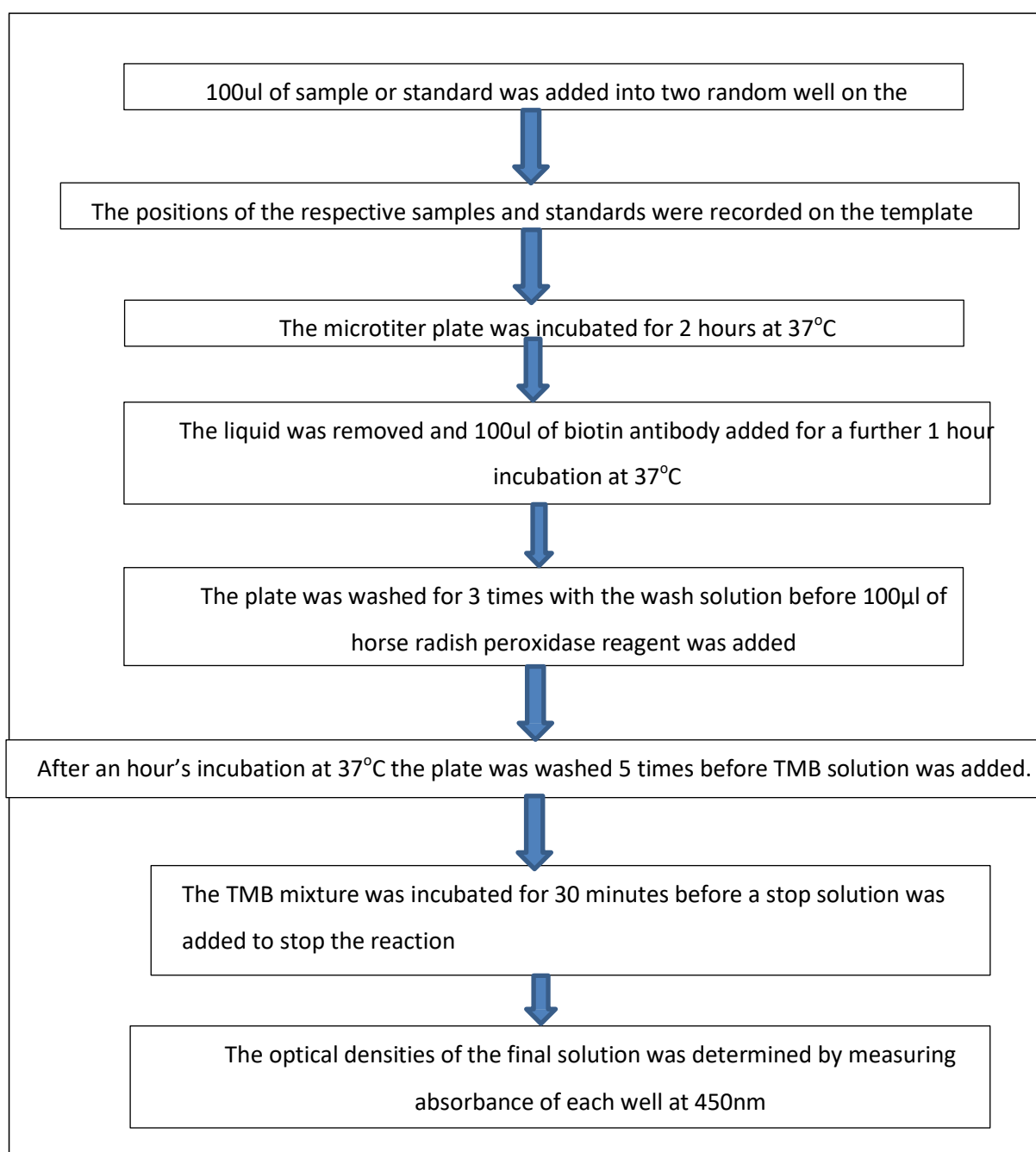
On the day of experiments samples were taken from the -70°C freezer and thawed and brought to room temperature. Once thawed the samples were spun down at 100g for 5 minutes before being used to assay for Hepcidin. The reagents and standards were prepared before the assay was put up. Standards were prepared by making doubling dilutions of the initial standard preparation. The initial standard preparation was made by reconstituting the standard with 1000µl of sample diluent to make a



Hepcidin concentration of 300ng/ml. A two-fold dilution was made from the initial standard and this was repeated for a further 5 times to make 7 standard solutions with Hepcidin concentrations varying from 300ng/ml to 4.69ng/ml. An additional vial containing sample diluent only was also used as a blank.

After all the reagents and standards were prepared, the Hepcidin assay for the pre-iron IDA group was put up by running both the samples and standards in duplicate on a 96 well microtiter plate. These were randomly placed in the wells of the microtiter plate and the positions noted on a template. The procedure for running the test was as illustrated in Figure 3.1.

**Figure 3.1: Hepcidin assay method showing step by step procedure for performing the ELISA test.**



After carrying out the experiments for Hepcidin determinations as described in Figure 3.1 above the optical density readings were used to extrapolate the Hepcidin concentration results from the standard curve. The standard curve was constructed using the antilogarithms of the known Hepcidin concentrations and the respective optical densities.

#### 3.3.4.2. Full blood count analysis

Samples for full blood count analysis were tested on the day of collection using the Pentra XL80 five-part differential analyser. However, the analyser has a 48-hour post draw stability which allows it to produce valid results from a sample up to 2 days old (ABX user manual n.d).

The principles of measurement for this analyser are discussed earlier in the previous chapter. On the day of sample collection, the samples were collected from qualifying participants and placed in a temperature controlled environment. Samples were stored at 4-8°C before processing. They were then transported to the National University of Science and Technology (NUST) for processing. During processing 53µL of properly mixed whole blood was aspirated into the analyser. The whole blood was obtained from samples collected in EDTA specifically to assess full blood count parameters for the Hepcidin study.

In the FBC analyser the aspirated blood was split into different counting/analysis chambers utilising the multi-distribution sampling system (MDSS) technological capability of the analyser (ABX technical manual 2002). A full range of results were generated from the respective counting/ analysis chambers. FBC parameters produced include haemoglobin, MCV, MCH, MCHC, platelets and RDW of which some of them were recorded on the data collection tool. The FBC results were obtained for both the pre- iron and post- iron groups.

#### 3.3.4.3. Serum iron assays

Serum iron results were obtained using the FerroZine colourimetry method for quantitative determination of iron. The assays were performed in batches using serum that was collected and stored at -70°C. According to the manufacturer's instructions as indicated in appendix 1, samples could be stored for a week in a refrigerator at 2-8°C (CHRONOLAB n.d.). In this case the samples were however frozen which allowed them to be stable for longer durations. In this study the samples were frozen and stored for at most 6 weeks before being assayed.

The principle of the FerroZine calorimetric method for determination of serum iron is described earlier in the previous chapter. Samples collected from deferred anaemic

blood donors were assayed for serum iron to confirm the presence of iron deficiency. The same group of participants were followed up after administration of iron therapy, mainly oral iron tablets, and another set of samples collected. The second set of samples was assayed for serum iron to assess the effect of previously administered iron therapy. The procedure for performing the serum iron test is as described earlier in the previous chapter.

### **3.4. Data management**

Data collected in the form of participant details was anonymised by the use of unique identification numbers. Other forms of data collected were results from the laboratory experiments. The results included haemoglobin (Hb) levels, Hepcidin values as well as serum iron levels. These results were collected from both the reference normal range group and the iron deficiency anaemia group. The results were collected using a data collection tool which captured all the raw data. Original readings from the ELISA plate reader were printed on a template and where the sample numbers were also captured. This was transferred to an excel spreadsheet which was used as the secondary data collection tool. The data was then entered into a statistical software for cleaning and analysis.

The data generated from the two studies was analysed using statistical package for the social sciences (SPSS) version 24. Both the data analysis and cleaning was performed using SPSS. Data cleaning was performed by paying attention to data cleaning items such as participant identification numbers, missing values, skip patterns as well as implausible values for the analytes that were tested.

The participant identification numbers were put in a frequency table to check if there were any duplicate entries. Missing values are usually given a code in SPSS. This however was not done for the data generated in both the reference normal range and the iron deficiency group. Missing data in SPSS was investigated by going back to the original data set to confirm if the values were not erroneously omitted. Once confirmed that the values are genuinely missing the default series mean method was employed to replace the missing values. With this method, the mean value of the analyte in question was calculated and the value was used to replace the missing data in that

series. The same approach was also used with implausible values after accounting for the quality of samples. Sample validity and sample integrity is paramount to generating useable data therefore the quality of samples was assessed before and after performing the experiments to generate data.

#### **3.4.1. Assessment of normality**

The distribution of the generated data was assessed in two ways by visually inspecting the histograms generated and also by statistically analysing descriptive statistics in SPSS. Visual inspection has the advantage of making judgement based on the graphical interpretation of the histograms or any other such graphical presentations. Furthermore, visual inspection enables a good judgement to be made where statistical tests prove to be over sensitive or even under sensitive. Visual assessment however is more reliable with experienced individuals thus both approaches were used in this project.

Statistical analysis of descriptive statistics was performed using the 'explore' command in SPSS. In instances where the data failed the normality test data transformation was performed and tested for normality again. Common transformations include converting variables to their logarithmic values or using square roots of the dependent variables.

#### **3.4.2. Power calculation**

A power calculation was performed to ensure a sufficient sample size was determined by the sample size calculation. In calculating the desired power, the standardised difference (Sdiff) was obtained using the following formula.

$$\text{Sdiff} = \frac{\text{Difference between means}}{\text{Population standard deviation}} \text{ (Jones et al., 2003).}$$

The minimum desired power was set at 0.80 and the sample size was extrapolated from the Altman's nomogram for sample calculation using power and standardised differences (Altman 1981). A copy of the nomogram is included as Appendix 8 in the appendices section.

The difference between means was obtained by subtracting mean Hepcidin concentrations of health non-IDA participants as obtained from Sdgou et al., (2015), Gonzo et al., (2017) and Pasricha et al., (2011) from the mean Hepcidin concentrations of participants with IDA.

Means for IDA participants were obtained from a study by Takasawa et al., (2015) and this current study. The means are tabulated in Table 3.1 below.

**Table 3.3: Mean Hepcidin concentrations obtained from other studies.**

Hepcidin means from non-IDA participants		Hepcidin means from IDA participants	
Source	Hepcidin (ng/mL)	Source	Hepcidin (ng/mL)
Sdgou et al., (2015)	45.60; 61.27; 47.58; 51.39; 55.38; 60.84	Takasawa et al., (2015)	10.8
Gonzo et al., (2017)	51.99	Current study	9.85
Pasricha et al., (2011)	28.5		
Average	50.32		10.33

The population standard deviation (SD) of 18.94ng/mL was used. This was obtained from previous work carried out on Hepcidin by Sdgou et al., (2015), Parischa et al., (2011) and Galesloot et al., (2011).

A standardised difference of >1.2 was obtained. Extrapolating from the nomogram, a sample size of 40 participants would give a power of >0.95 which was above the set minimum of 0.8 implying that the sample size was sufficient.

## CHAPTER 4

### 4. RESULTS

#### 4.0. Introduction

Results were obtained from the Reference Normal Range (RNR) group and the Iron Deficiency Anaemia (IDA) group. Data from the reference normal range group was used to establish Hepcidin normal values within the Namibian populace. Furthermore, data from the iron deficient group was used to study the quantitative behaviour of Hepcidin in both normal and inadequate iron states.

In both study populations, incomplete results were excluded from the study. Incomplete results were mainly due to the presence of data sets where either one or more results were missing for a particular blood sample. Another reason for missing data was the inability to obtain further samples from the participants. One sample in the reference normal range group had full blood count results but could not be tested for serum iron and Hepcidin. The blood sample was found to be haemolysed after thawing. Haemolysis interferes with spectrophotometric measurement of iron and Hepcidin therefore a complete data set could not be obtained, and this resulted in the blood sample being excluded.

In the IDA study population there was a notable degree of sample attrition as participants failed to turn up for subsequent blood sample collections. Due to the nature of the sampling method; sequential sampling, follow up samples were collected 3 months after the initial blood sample collection. As a result of no-shows, a further 5 participants were recruited to account for sample attrition as some participants did not turn up for follow up samples after initiating iron treatment. Recruitment of additional participants allowed for the collection of data from a complete set of samples as determined by the sample size calculations performed for this participant group.

## 4.1: Results from the Reference Normal Range (RNR) group

### 4.1.1 Demographic features

A total of 40 donors were recruited to participate using a multistage sampling method which comprised simple random sampling and stratified sampling. Donors were stratified according to whether they were eligible to donate on the day they presented at the blood transfusion service for donation. Those able to donate were randomly selected and recruited into the study between October 2015 and June 2016. Thirty-nine (39) samples from the recruited participants were tested. The other blood sample could not be tested due to sample validity issues. Age, serum iron and some haematological values of the tested parameters are summarised in Table 4.1

**Table 4.1: Hepcidin, serum iron levels and haematological values in the reference normal population group**

Variable	Average	Standard deviation (SD)
Age (years)	40 years	10.511
Haemoglobin	143.56g/L	12.92
Mean corpuscular volume	92.53 FL	4.93
Hepcidin	51.99ng/ml	17.44
Serum iron	91.32µg/dL	16.78

Serum iron values for this sample ranged from 69µg/dL to 150µg/dL with a median value of 91.5µg/dL. The mean value for serum iron (91.32µg/dL) gives a good indication of iron bioavailability.

The mean Hepcidin concentration for the whole study population was 51.99ng/ml. Thirty-nine (39) samples were tested with 19 females and 20 males. The haemoglobin values ranged from 112g/L to 168g/L for males and 125g/L to 157g/L for females all of which were normal for both genders. Compared to the haemoglobin values, the Hepcidin values were much spread out.

These are illustrated in Figure 4.1 below which shows the distribution of Hepcidin values in the reference normal range group.



**Figure 4.1: Values and distribution of Hepcidin levels in the reference normal range group.**

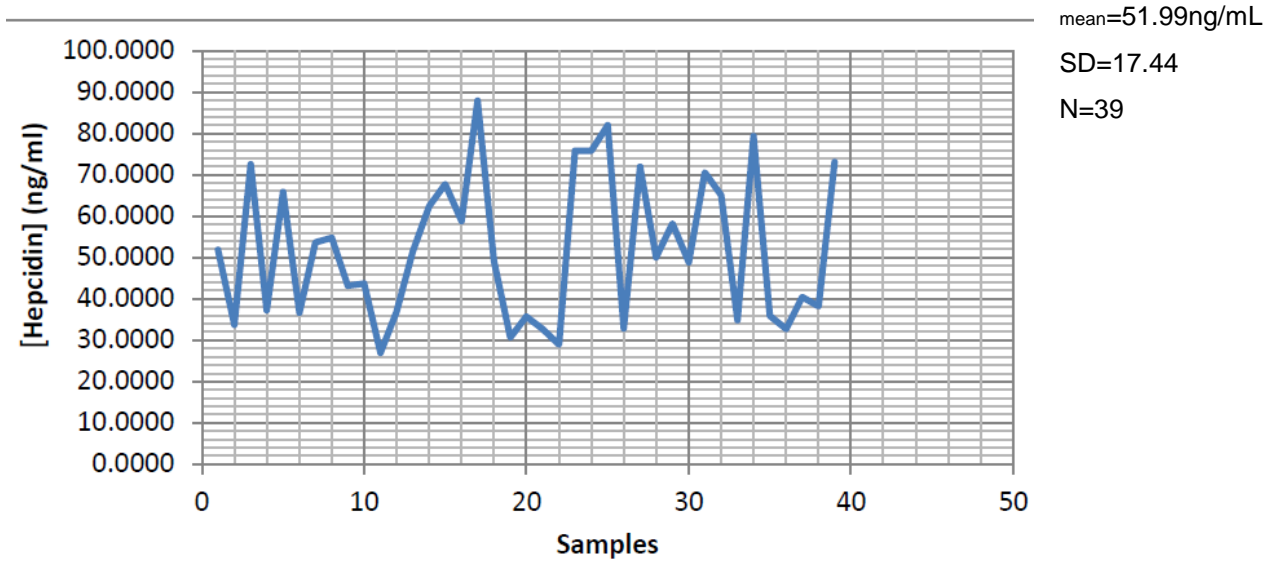


Figure 4.1 indicates that the lowest and highest hepcidin values in the reference normal range group. Most of the values were above 40ng/ml with 9 (~25%) of them having Hepcidin values of at least 70ng/mL.

#### 4.1.2. Data analysis

Data analysis for this participant group was performed to obtain Hepcidin reference normal ranges within this study group. Confidence intervals and reference ranges were calculated using the following function as adopted from Altman (1991).

$$rr = \bar{x} \pm z_{(1-\alpha)/2} s$$

$$se = \sqrt{\frac{s^2}{n} + \frac{z_{(1-\alpha)/2}^2 s^2}{2n}}$$

$$ci = rr \pm z_{\alpha/2} se$$

Formula for reference ranges calculation: Altman 1991; pp.421-422.

This was performed using the following:

X bar= normal range

N= sample size

S= sample standard deviation

Se= se is the standard error of the reference range limits ci

= confidence interval for the reference range limits

$z$  = a quantile from the standard normal distribution and

$c$  = % range coverage/100 (in this case 0.95 for a 95% reference range).

The reference normal range for Hepcidin in this study was 17.80-86.18ng/mL for the entire study population at 95% which is within 2SDs. The reference range of normal at 99% was 34.55-69.43ng/ml which is within 1SD. For the work on IDA a reference range within 2SD was adopted as the normal reference range for Hepcidin in the population under study. For the two genders, the ranges for the females were 17.19-91.24ng/mL and for males it was 18.23-81.54ng/mL both at 95%. Other statistical values are indicated in Table 4.2 below.

**Table 4.2: Statistical values for Hepcidin and haemoglobin in the reference normal range population**

	Hepcidin ng/mL	Haemoglobin g/dL	Hepcidin ng/mL		Haemoglobin g/dL	
	Non-gender specific		Female	Male	Female	Male
<b>Mean</b>	<b>51.99</b>	<b>14.36</b>	<b>54.21</b>	<b>49.89</b>	<b>13.83</b>	<b>14.86</b>
Median	50.02	14.10	51.51	49.02	13.70	14.45
Mode	32.67	14.10	75.83	N/A	13.70	14.10
Standard Deviation	17.44	1.28	18.89	16.15	0.91	1.39
Minimum	26.91	12.50	26.91	28.89	12.50	12.60
Maximum	87.90	16.80	81.97	87.90	15.70	16.80
<b>95% Lower Limit</b>	<b>17.80</b>	<b>11.86</b>	<b>17.19</b>	<b>18.23</b>	<b>12.04</b>	<b>12.14</b>
<b>95% Upper Limit</b>	<b>86.18</b>	<b>16.86</b>	<b>91.24</b>	<b>81.54</b>	<b>15.61</b>	<b>17.58</b>
CI for Lower limit	12.33- 23.28	11.46- 12.26	8.69- 25.68	11.15- 25.31	11.63- 12.45	11.54- 12.75
CI for upper limit	80.71- 91.66	16.46- 17.26	82.74- 99.73	74.46- 88.62	15.20- 16.02	16.97- 18.19

The results in Table 4.2 above show lower confidence levels (LCL) and upper confidence levels (UCL) for both the lower and upper limits. The standard deviation of Hepcidin for females (18.89) was higher than that obtained for males (16.15) despite males having more samples than females.

## **4.2 Results for the Iron Deficiency Anaemia (IDA) group:**

A total of forty participants were required for the study but forty-five prospective donors were recruited to compensate for those who dropped out of the study. The participants were prospective donors who failed the haemoglobin  $\text{CuSO}_4$  test. They were recruited into the study to assess the quantitative behaviour of Hepcidin in anaemia. This was done by testing the participants' blood samples for full blood count, serum iron and Hepcidin levels. These participants were followed up after iron treatment and tested for the same parameters.

Results in this section are presented in three sub sections namely:

- results from participants with iron deficiency anaemia before iron therapy,
- results from the same participant group after iron therapy and
- a comparison of the two groups.

Where possible, results from the normal healthy participants group were also combined with ones from the iron deficiency anaemia group. This was done to obtain a full picture of the characteristics of the analytes being investigated. The comparison mainly focused on the analytes of interest such as the similarities or differences of Hepcidin, serum iron and full blood count parameters. The full blood count parameters which were looked at included haemoglobin (Hb), red cell distribution width (RDWcv) and the red cell indices such as mean corpuscular volume (MCV) and mean corpuscular haemoglobin concentration (MCHC).

### **4.2.1. Results before treatment with iron**

Serum iron levels were measured in the IDA group and it was noted that the majority of participants in this group were iron deficient. The histogram in Figure 4.2 below shows the distribution of serum iron levels in the IDA participant group.

**Figure 4.2: Histogram of serum iron levels in the IDA participant group before treatment with iron supplementation.**

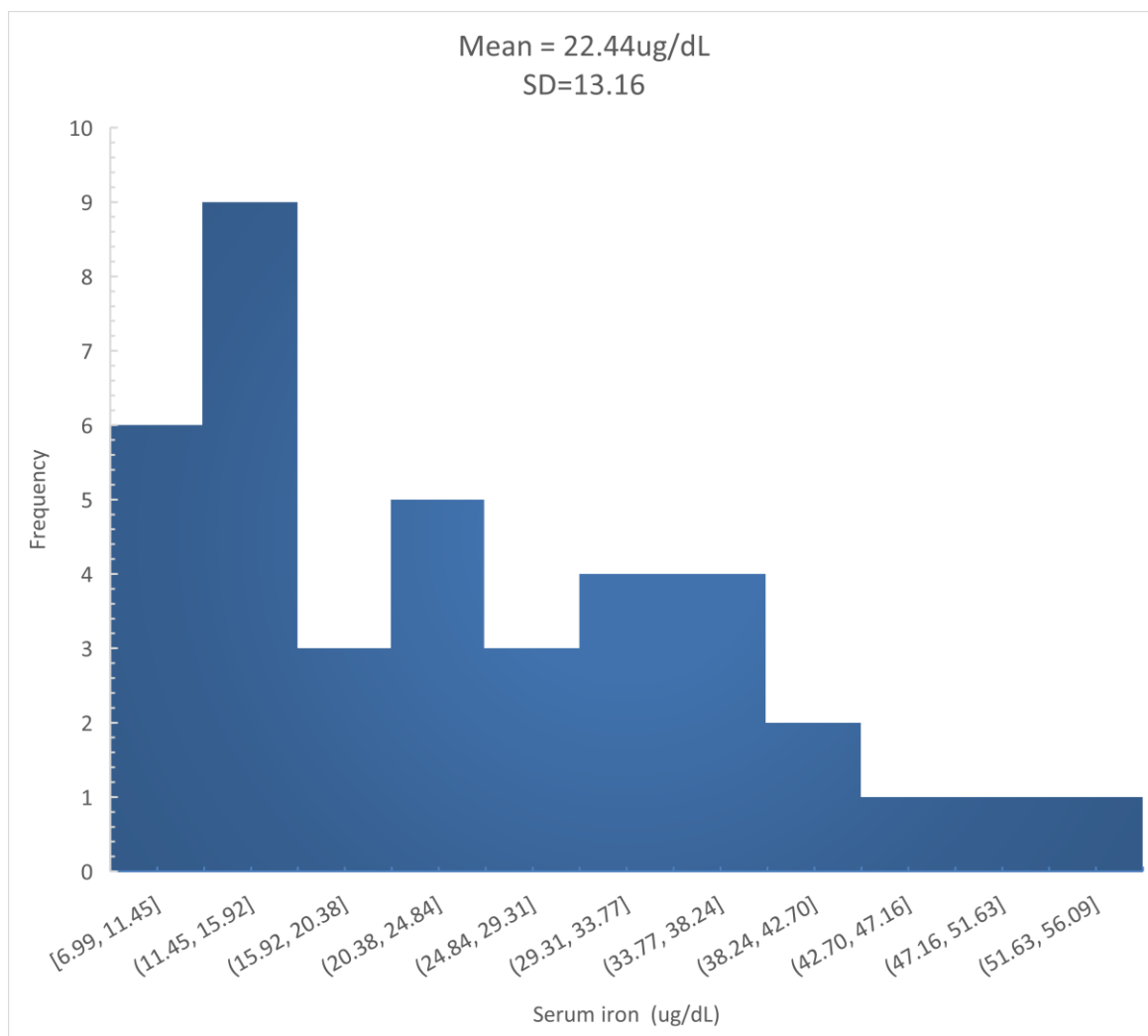
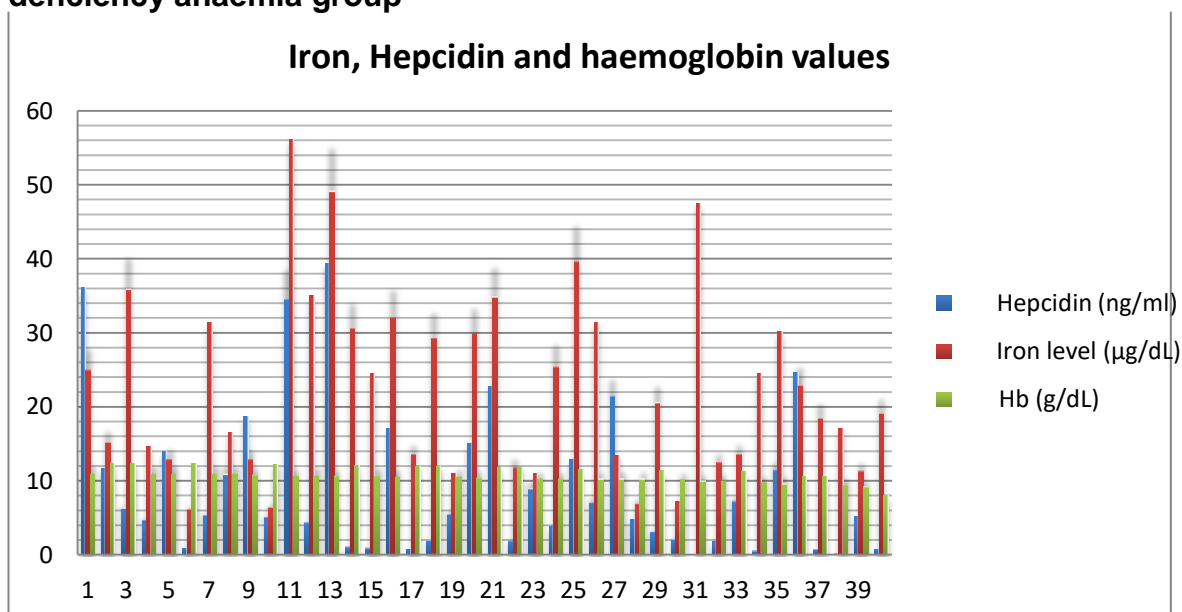


Figure 4.3 below, shows the same serum iron levels in relation to Hepcidin and haemoglobin values in the iron deficiency anaemia group. These values were obtained before participants commenced treatment with iron in order to have a clear understanding of the baseline values in iron deficiency anaemia.

**Figure 4.3: Graph showing iron, Hepcidin and haemoglobin values in the iron deficiency anaemia group**



.From the chart in figure 4.3 above it can be noted that the serum iron level values ranged from 6.99µg/dL to 56.09µg/dL in this study group. The haemoglobin and Hepcidin values ranged from 80g/L to 125g/L and 0.10ng/mL to 39.40ng/mL respectively in the same study group. These values confirm the presence of iron deficiency anaemia with some level of certainty given low haemoglobin values in the presence of suppressed serum iron and Hepcidin levels.

#### 4.2.1.1. The effect of iron and the effect of Hepcidin on Haemoglobin

A correlation analysis was performed to evaluate the strength of relationships between serum iron, Hepcidin and haemoglobin. These relationships were assessed in the presence of inadequate bioavailable iron and the results are tabulated in Table 4.3 below.

**Table 4.3: Individual effects of serum iron (a) and Hepcidin (b) on Hb: An analysis in IDA patients before commencing iron therapy**

	4.3a		4.3b	
Correlation	Serum iron	Log serum iron	Hepcidin	Log Hepcidin
<i>r</i>	0.10	0.03	0.26	0.37
<i>r</i> square	0.01	0.01	0.07	0.13
Observations	40	40	40	40
p-values	0.54	0.86	0.10	0.02

The correlation analysis above shows the associations between serum iron, Hepcidin and haemoglobin levels. Correlation analysis and multiple regression are different, but they complement each other. Correlation analysis describes the linear relationship between two variables, it focuses on the strength of the relationships. It is pertinent to note that correlation and causation are different. Regression analysis can predict the relationships between two variables, it can show how one variable changes with the other. Furthermore, multiple regression can be used to predict how one dependent variable changes in line with other independent variables. To compliment the correlation analysis results above, outputs from a multiple regression analysis are presented below. Table 4.4 shows a summary of the values obtained after performing multiple regression analysis between haemoglobin and other variables. The independent variables investigated were Hepcidin, serum iron, red cell indices and red cell count.

**Table 4.4: Multiple regression analysis showing the effect of various factors on haemoglobin**

<b>Multiple R</b>	<b>77.05%</b>
<b>R-Square</b>	<b>59.36%</b>
<b>R-Square Adjusted</b>	54.64%
<b>S (Root Mean Square Error)</b>	1.48

From the multiple regression Table 4.4 above, it can be noted that the  $r$  overall (highlighted in red) is 0.7705 which is reasonably close to 1. The  $r$  overall, usually termed multiple R shows the combined effect of the named variables to haemoglobin. The eight independent variables were RBC, Hct, MCV, MCH, MCHC, serum iron, Hepcidin and RDWcv. The obtained value of 77.05% show that all the eight variables combined have a reasonable effect towards the variability of haemoglobin. This indicates that experimental results from this study looked at most of the significant players in anaemia. This also helps to explain the low haemoglobin correlation values with the two variables; serum iron and hepcidin as seen in Tables 4.3a and 4.3b. These two variables are not the only parameters influencing haemoglobin levels.

#### 4.2.1.2. The effects of various factors on Hepcidin

Having looked at how haemoglobin is associated with other haematological parameters that are relevant to iron deficiency anaemia in Table 4.4, it is also important to describe the behaviour of Hepcidin in relation to the full blood count parameters. Table 4.5 below shows the correlation values of Hepcidin with red cell indices and serum iron. The correlations were performed on data obtained from both the IDA and the normal healthy adult participant groups.

**Table 4.5: Effect of various parameters on Hepcidin, a correlational analysis**

	Hb	RBC	HCT	MCV	MCH	MCHC	RDWcv	Iron	Hepcidin
Hb	1								
RBC	0.77	1							
HCT	0.94	0.84	1						
MCV	0.53	0.02	0.55	1					
MCH	0.44	-0.15	0.35	0.87	1				
MCHC	-0.25	-0.19	-0.31	-0.26	-0.09	1			
RDWcv	-0.22	-0.23	-0.18	0.04	-0.02	-0.07	1		
Iron	0.74	0.58	0.78	0.52	0.41	-0.27	-0.16	1	
Hepcidin	0.62	0.46	0.65	0.48	0.36	-0.21	-0.08	0.71	1

Table 4.5 shows the association of Hepcidin with other parameters, highlighted in yellow. In that row it can be seen that iron has the highest correlation value ( $r=0.71$ ) with Hepcidin followed by haematocrit (HCT) ( $r=0.65$ ) and then haemoglobin ( $r=0.62$ ) compared to all the other parameters. The iron-Hepcidin correlation can be regarded as strong implying that there is a strong positive association between iron and Hepcidin. This confirms that the behaviour of Hepcidin is positively influenced by iron in situations where iron deficiency is not present as well as where there is not enough iron available for normal physiological functions. It therefore confirms that it is possible to assess the trend in iron bioavailability using Hepcidin levels.

Full blood count parameters (Hb, RBC, Hct, MCHC and RDWcv) that are highlighted in blue on Table 4.5 have moderate to very strong positive associations with each other. These are RBC and Hb ( $r=0.77$ ); RBC and Hct ( $r= 0.84$ ) and Hb and Hct ( $r= 0.94$ ). The serum iron levels also have strong correlations with Hb, RBC, HCT and MCV. These are highlighted in green on Table 4.5 above.

It is interesting to note that MCHC and RDWcv have negative correlations with almost all the parameters. The associations are however weak ( $r > -0.25$ ).

Full blood count parameters, in particular haemoglobin levels remain the hallmark for defining anaemia as such these results are important in diagnosis of iron deficiency anaemia. It is suggested that the full blood count parameters are not replaced but are complemented with Hepcidin testing. Other parameters which are currently in routine use to assess iron bioavailability could benefit from the introduction of Hepcidin testing because of Hepcidin's response. To illustrate this further, an excerpt from the multiple regression analysis output is shown in Table 4.6.

**Table 4.6 : Regression analysis data showing the effects of various factors on Hepcidin**

on Hepatoma

Multiple R	55.40%	
R-Square	30.70%	
R-Square Adjusted	12.80%	
S (Root Mean Square Error)	3.15	
Parameter	Coefficients	P-value
Hb	5.19	0.91
RBC	34.55	0.46
HCT	-5.14	0.76
MCV	7.35	0.33
MCH	-17.60	0.35
MCHC	14.70	0.26
RDWcv	0.51	0.52
Iron	0.34	0.01

From Table 4.6, it can be seen that the  $r$  overall (multiple R) of 0.554 demonstrates a fair effect of the combined eight variables on Hepcidin. The  $R^2$  value, also known as the coefficient of multiple determination, shows that the eight factors combined contribute 30.7% to the quantitative behaviour of Hepcidin.



From the coefficients values in Table 4.6 above, it means that for every  $1 \times 10^{12}$  cells/L increase in red blood cells (RBC) Hepcidin will increase by 34.55ng/mL while holding the remaining parameters constant. Equally for every 1g/dL increase in haemoglobin Hepcidin will increase by 5.19ng/mL if we hold the remaining parameters constant. These are reasonable effects which however needs further explanation in light of the role of iron bioavailability in red cell formation. In holding other parameters constant while increasing the haemoglobin level, it means iron mobilisation from iron stores or absorption from the duodenum has to be increased to cater for iron usage in haemoglobin production. Hepcidin expression is subsequently upregulated in response to the correcting anaemia condition.

The effect of red blood cells and haemoglobin on Hepcidin is however dependant on iron because Hepcidin regulates iron and iron is used in the production of haemoglobin. The probability values in Table 4.6 above can help to illustrate that. On all the parameters, only iron has a statistically significant effect on Hepcidin ( $p=0.01$ ), the rest are not close to 0.

The average haemoglobin level in this study group was 11.60g/dL with a mode of 12.30g/dL of which both figures indicate reduced haemoglobin levels. Since these are two of the three measures of central tendency we can associate the presence of anaemia in this study group given the low values of the mean and the mode. To clarify this further, other values for iron and hepcidin are indicated in Table 4.7.

**Table 4.7: Descriptive statistics values for the pre-iron therapy group**

Pre-iron group	Hb (g/dL)	Iron (ug/dL)	Hepcidin (mg/mL)	Log Hepcidin	Log Iron
Mean	11.6	24.437	9.848	0.673	1.323
Standard Error	0.128	2.081	1.684	0.101	0.039
Median	11.8	22.387	5.834	0.766	1.350
Mode	12.3	14.657	N/A	N/A	1.166
S D	0.808	13.159	10.650	0.636	0.249
Variance	0.653	173.166	113.425	0.404	0.062
Range	4.5	49.103	39.301	2.617	0.905
Minimum	8.0	6.988	0.095	-1.022	0.844
Maximum	12.5	56.092	39.396	1.595	1.749
Count	40	40	40	40	40
C. I (95.0%)	+/- 0.258	+/- 4.209	+/- 3.406	+/- 0.203	+/- 0.080

#### 4.2.2. Results of the IDA group after treatment with iron.

The same group of iron deficiency anaemia participants as discussed in section 4.2.1 underwent treatment with iron before being tested again at 3 months. The mean values for the relevant parameters were obtained from respective paired t- tests and are shown in Table 4.8.

**Table 4.8: Comparison of Haemoglobin, Hepcidin and iron values pre-and post-iron treatment as obtained from the Paired Two Sample for Means t-Test.**

Variable	Pre-iron treatment	Post iron treatment	p-value from the respective paired t-test
	Average (SD)	Average (SD)	
Age (years)	31 (+/- 12.89)	31 years 9+/-12.89)	N/A
Haemoglobin (g/L)	116.00 (+/-8.08)	132.78 (+/-8.99)	$2.6 \times 10^{-20}$
Hepcidin (ng/ml)	9.848 (+/- 10.65)	79.851 (+/- 40.154)	$8.6 \times 10^{-13}$
Serum iron (µg/dL)	24.44 (+/- 13.16)	80.112 (+/- 9.474)	$4.4 \times 10^{-23}$

The average haemoglobin levels were much higher after treatment with iron, an indication that administration of iron had a positive effect on the haematological state of the participants. Hepcidin mean also went up together with serum iron values signalling an increase in iron bioavailability. To assess the utility of Hepcidin as a diagnostic test in assessing iron bioavailability a ROC analysis was performed. Table 4.9 below shows sensitivity and specificity calculations done for the Hepcidin test. The values of the predictions are also included in the tables together with the formulae used to calculate them.

**Table 4.9: Predictive values for absolute Hepcidin levels in iron deficiency anaemia**

Test	Disease		n	Absent	n	Total
	Present					
Positive	True Positive	a=34	False Positive	c=0	a + c = 34	
Negative	False Negative	b=6	True Negative	d=40	b + d = 46	
Total		a + b = 40			c + d = 40	

Statistic	Formula	Value	95% CI
Sensitivity	$a/(a+b)$	85.00%	70.16% to 94.29%
Specificity	$d/(c+d)$	100.00 %	91.19% to 100.00%
Positive Likelihood Ratio	$\frac{\text{Sensitivity}}{(100-\text{specificity})}$	N/A	
Negative Likelihood Ratio	$\frac{(100-\text{sensitivity})}{\text{Specificity}}$	0.15	0.07 to 0.31
Disease prevalence	$(a+b)/(a+b+c+d)$	50.00% (*)	38.60% to 61.40%
Positive Predictive Value	$a/(a+c)$	100.00% (*)	89.72% to 100.00%
Negative Predictive Value	$d/(b+d)$	86.96 % (*)	73.74% to 95.06%

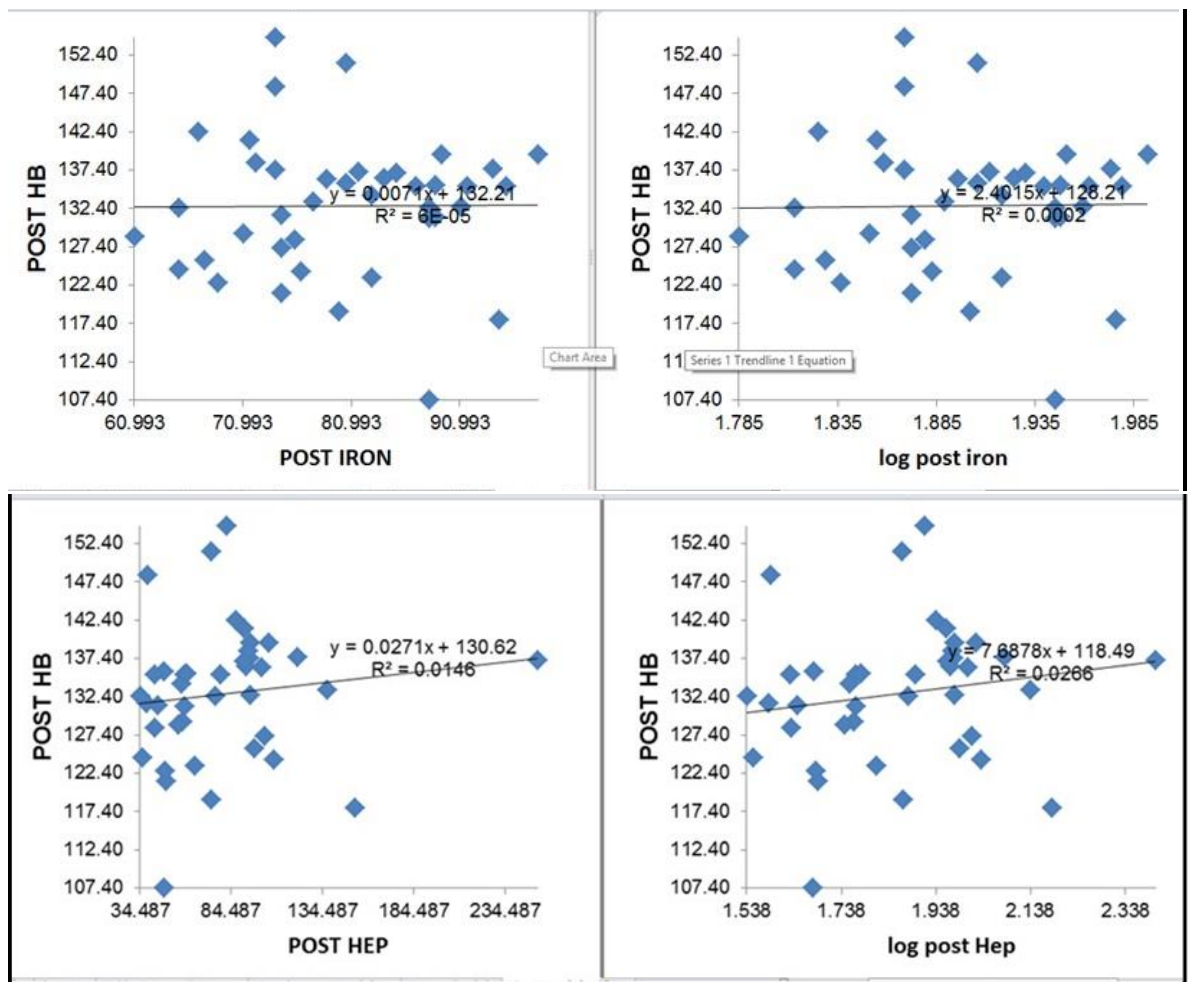
The favourable results shown in Table 4.9 above show that Hepcidin is a potentially useful diagnostic test. The correlation analysis on the post iron treatment group would also help to ascertain the utility of routine Hepcidin testing in iron deficiency anaemia thus these are presented in Table 4.10 below.

**Table 4 . 1 0 : Serum iron, Hepcidin and haemoglobin correlation analysis in IDA patients after iron therapy**

<b>Pearson Correlations</b>	<b>Post Hb</b>	<b>Post iron</b>	<b>Log Post iron</b>	<b>Post Hepcidin</b>	<b>Log Post Hepcidin</b>
Post Hb	1	<b>0.01</b>	<b>0.02</b>	<b>0.11</b>	<b>0.15</b>
Post iron		1	<b>1.00</b>	0.22	0.27
Log Post iron			1	0.22	0.27
Post Hepcidin				1	<b>0.94</b>
Log Post Hepcidin					1

The two analytes, serum iron and Hepcidin have very weak correlations with haemoglobin levels as shown by the highlighted r values in the correlation matrix in Table 4.10. Hepcidin nevertheless has a better correlation than iron. The use of logarithmic values of Hepcidin tends to give better results. To demonstrate this further, scatter plots showing the different correlations with haemoglobin values after treatment with iron are shown in Figure 4.4 below. The four scatter plots indicate the relationships between haemoglobin (HB) & iron and haemoglobin & Hepcidin (HEP). The figure also shows the relationship between haemoglobin and logarithmic values of iron and Hepcidin.

**Figure 4.4: Scatterplots showing the relationship between haemoglobin and other variables**

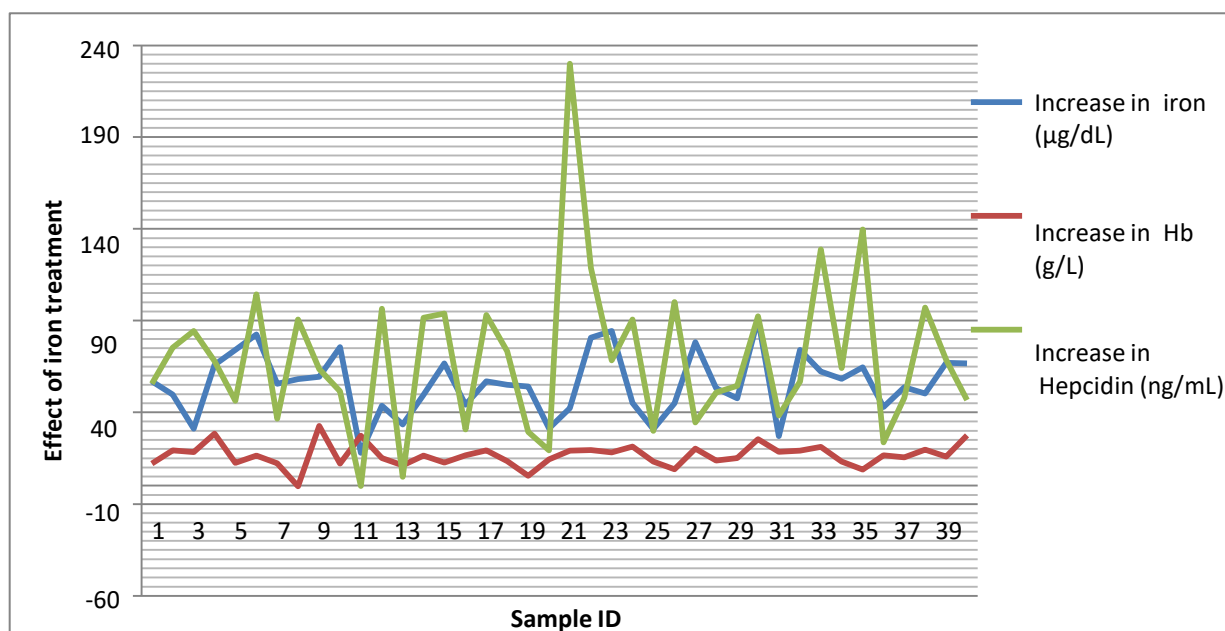


From the scatterplots in Figure 4.4 above it can be shown that log Hepcidin values offer a better relationship with haemoglobin in the IDA participants who had undergone treatment with iron. The trend lines for serum iron values are almost horizontal. Compared to the Hepcidin trend lines, Hepcidin appears to have an association with haemoglobin. Logarithmic values of Hepcidin have a slightly better positive correlation than absolute Hepcidin values.

Generally, the behaviour of Hepcidin in both iron replete and iron deplete states is consistent with the anaemic status of the samples under study. The Hepcidin levels are depressed in iron deplete states. On the contrary, Hepcidin levels return to normal in iron replete states. Its performance however is strongly linked to iron bioavailability and it is pertinent to look at how serum iron, Hepcidin and

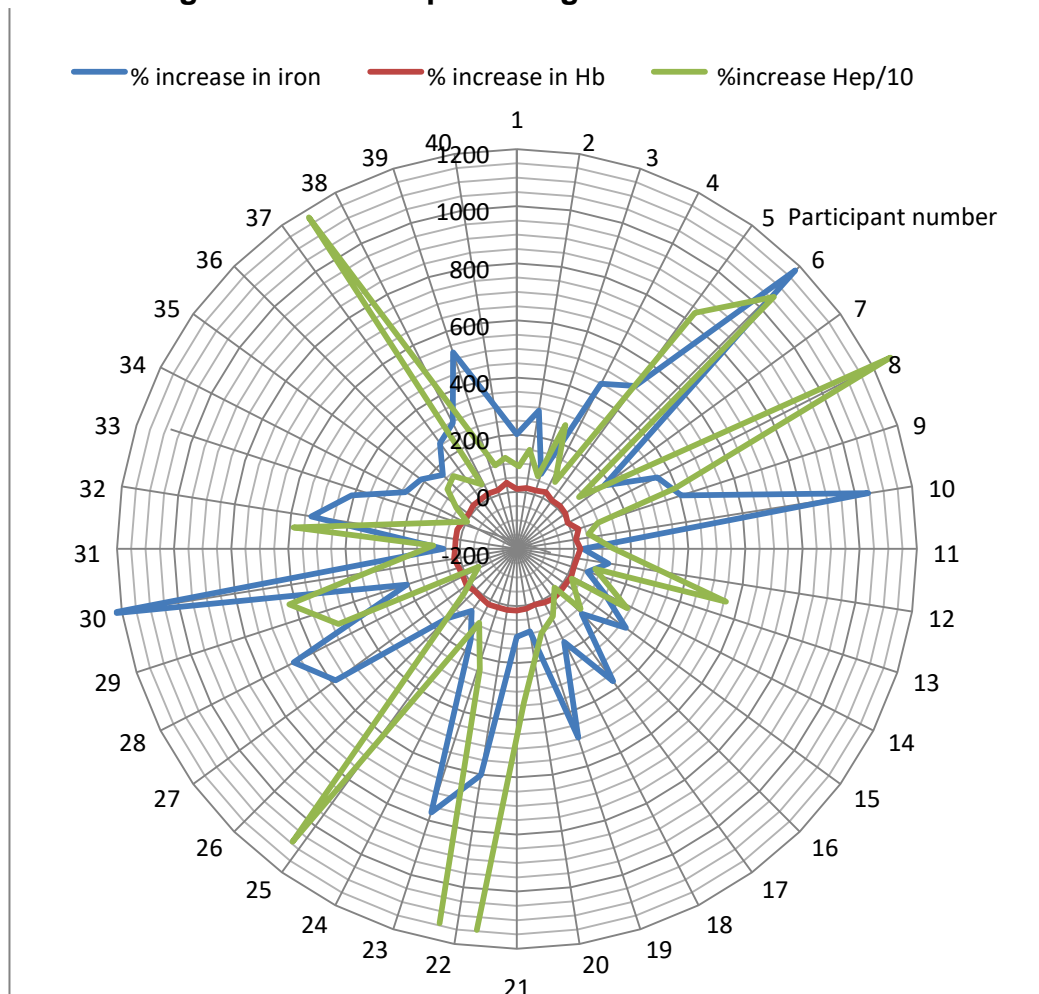
haemoglobin levels were affected by treatment with iron in this participant group. Figure 4.5 and 4.6 illustrate this effect.

**Figure 4.5: Chart showing the effect of iron treatment on actual serum iron, Hepcidin and haemoglobin levels**



In Figure 4.5 above an increase in both haemoglobin and serum iron was noted. In most cases the trends in the magnitude of increase are similar for both haemoglobin and serum iron illustrating that haemoglobin levels increases with iron bioavailability in IDA. The increase in Hepcidin values however is more pronounced than serum iron. The percentage increase for Hepcidin is even much more pronounced with values above 1000% increase being common. The magnitudes of the increases are also shown in Figure 4.6 below as percentage increases in the three parameters.

**Figure 4.6: Chart showing the effect of iron treatment on serum iron, Hepcidin and haemoglobin levels as percentage increments.**



The line chart in Figure 4.5 and the radar chart in Figure 4.6 show that most participants responded positively to iron treatment.

The serum iron values increase was much higher than that of haemoglobin in all the participants who undertook iron treatment. The increase in Hepcidin levels was

however much higher than for both iron and haemoglobin. The increase in haemoglobin ranged from 5.7g/L to 14g/L and serum iron increases were between 18µg/dL to 91µg/dL. Hepcidin increments were much more pronounced ranging from -0.19ng/mL to 229ng/mL. The average percentage increase in iron, haemoglobin and Hepcidin was 353.2%, 14.6% and >5000% respectively. On the chart in Figure 4.6 the percentage increase of Hepcidin was plotted as a tenth ( $10^{-1}$ ) of the percentage increase to enable it to fit on the same gridlines. This demonstrates the vast difference in percentage increments between serum iron and Hepcidin levels.

#### 4.2.3. Comparative analysis of pre-and post-iron treatment results

The comparative analysis of pre-and post-iron treatment results in Table 4.11 help us to study Hepcidin trends found in the prognosis of iron deficiency anaemia. The haemoglobin, iron and Hepcidin values changed as a result of iron treatment given to participants. The mean values for haemoglobin, Hepcidin and serum iron in Table 4.11 give a general idea of how these parameters changed in the different situations.

**Table 4.11: Mean values for Hb, Hepcidin and serum iron in different participant groups**

	<b>Cut off values</b>	<b>Participant group</b>		
Value	Lower limit of normal	Reference normal	Pre-iron treatment	Post-iron treatment
Haemoglobin (g/L)	118.00	143.60	116.00	132.70
Hepcidin (ng/mL)	17.80	51.99	9.845	79.85
Log Hepcidin	1.54	1.60	0.67	1.86
Serum iron (µg/dL)	58.40	91.30	24.40	80.10

The values for the three participant groups were as expected when compared to the cut off values for haemoglobin, Hepcidin and serum iron.



The pre- iron treatment group had values which were lower than the cut off (lower limit of normal) indicating the presence of iron deficiency anaemia. The haemoglobin levels in the post iron treatment group showed a marked increase compared to the pre-iron treatment group as reflected by the mean Hb levels in Table 4.11. The serum iron and Hepcidin mean values also show a similar trend with the mean increase of 70.00ng/mL and 55.7µg/dL for Hepcidin and serum iron respectively. The increase in the means for serum iron and Hepcidin were related to the rise in haemoglobin levels. It is therefore useful to take a closer look at the distribution of the change in haemoglobin levels ( $\Delta$  Hb) in the studied samples by looking at Figure 4.6 in conjunction with Table 4.11 showing the correlation data. The correlations in Table 4.12 refer to the pre-and post-iron treatment states.

**Table 4.12: Correlations between Hepcidin, serum iron,  $\Delta$  Hepcidin,  $\Delta$  serum iron and haemoglobin levels**

CORRELATION	<i>PRE-HB</i>	<i>POST IRON</i>	<i>PRE-IRON</i>	<i>POST HB</i>	<i>PRE-HEP</i>	<i>POST HEP</i>	<i>delta HEP</i>	<i>delta IRON</i>	<i>delta Hb</i>
PRE-HB	1								
POST IRON	-0.03	1							
PRE-IRON	0.10	-0.10	1						
POST HB	0.74	0.02	0.05	1					
PRE-HEP	0.26	-0.00	0.46	0.24	1				
POST HEP	0.11	0.22	-0.06	0.11	-0.02	1			
delta HEP	0.04	0.21	-0.17	0.05	-0.28	0.97	1		
delta IRON	-0.09	0.64	-0.83	-0.03	-0.36	0.17	0.25	1	
delta Hb	-0.21	0.06	-0.06	0.50	0.01	0.03	0.02	0.08	1

## CHAPTER 5

### 5. DISCUSSION

#### 5.0. Introduction

The research was aimed at investigating the behaviour of Hepcidin under different experimental conditions. Hepcidin together with other analytes, such as serum iron and full blood counts, were assessed in normal adult participants as well as participants with iron deficiency anaemia. The use of different conditions has helped in establishing a reference normal range for use in this study and in clarifying the potential usefulness of Hepcidin in iron deficiency anaemia.

#### 5.1. Reference Normal Range group

Hepcidin reference normal ranges are useful as baseline information for the elucidation of the properties of this molecule in both iron deficient and iron replete states. Establishment of the reference normal ranges made it possible to assess the usefulness of hepcidin values in iron deficiency anaemia in this study. The reference normal range values established in this study were 17.80ng/mL to 86.18ng/mL without stratifying for gender. After stratification the reference normal range values were 17.19ng/mL to 91.24ng/mL for females and 18.23ng/mL to 81.54ng/mL for males. The reference normal range participant group provided sufficient data that is adequate to allow for this study on Hepcidin in iron deficiency anaemia to take place in the adult population.

Hepcidin values in the reference normal range (RNR) group ranged from 29.80ng/mL to 87.80ng/mL in the entire participant group. As shown in Table 4.1, the standard deviation of Hepcidin for females (18.89) was higher than that obtained for males (16.15) despite males having more samples than females. This demonstrates that the Hepcidin values for males were more concentrated around the mean compared to values obtain from female participants. It is important to note that for the entire study population the standard deviation of 17.44 obtained for Hepcidin in this study is slightly lower but consistent with what was obtained in similar studies

(Galeslout et al., 2011; Sdogou et al., 2015) that were used to calculate the sample size for this study.

For haemoglobin determinations, the minimum value was 125g/L which indicates that no anaemic individuals were recruited in this study group. The range for haemoglobin values were from 125.00g/L to 157.00g/L for females and 126.00g/L to 168.00g/L for males which is normal for the adult population. The mean, mode and median values for haemoglobin were close to each other. These values are measures of centrality which might mean the haemoglobin values in the study group are close to each other and this is supported by the low standard deviations for haemoglobin as indicated in Table 4.2.

The reference normal ranges established for the Namibian blood donor population are consistent with those established elsewhere (Parischa et al., 2011; Galeslout et al., 2011; Sdogou et al., 2015) albeit in different age groups. It is therefore reasonable to accept these as the true reflection of the Hepcidin levels in the blood donor population which in this case was used as a representation of the Namibian populace. The range of Hepcidin levels obtained in this study group was 60.99ng/mL for the entire study population. From established reference normal ranges elsewhere (Sdogou et al., 2015; Galeslout et al., 2011; Parischa et al., 2011) it is apparent that there is no suppression of Hepcidin expression in this group. These results together with serum iron level values determined in the same group, confirms the eligibility of the study group as a genuine group for the establishment on reference normal ranges. It is also important to note that the  $\pm 1$ SD value (17.44ng/mL) obtained for Hepcidin was slightly lower than what was obtained elsewhere in studies by Sdogou et al., (2015). This SD also indicates that the Hepcidin values obtained are reasonably spread around the mean.

The serum iron values were obtained in order to confirm the absence of iron deficiency in this group of participants. The serum iron levels ranged from 69 $\mu$ g/dL to 150 $\mu$ g/dL, results which showed that all the samples used had normal iron levels. These results are consistent with reference normal range values which are between 40 $\mu$ g/dL-155 $\mu$ g/dL for females and 55 $\mu$ g/dL-160 $\mu$ g/dL for males (Gomella and Haist 2007). The modal value for the serum iron levels was 96 $\mu$ g/dL and together with other values obtained, indicates good iron bioavailability in the

normal reference range group. Given the effect of iron levels on Hepcidin values, it is comforting to note that the Hepcidin reference ranges were derived from a population with normal serum iron levels.

The comparability of the study results to reference ranges established elsewhere makes it possible for us to use them in this study without incurring prohibitive costs associated with establishing comprehensive age and gender stratified reference normal ranges. This activity would however be advised when incorporating Hepcidin as a routine diagnostic test in practice. Alternatively, laboratories could opt to verify reference normal ranges established elsewhere with the intention of adopting the same values. Further reference normal range studies in different participant groups other than blood donors would also be useful in establishing reference normal ranges that can be used nationwide to run an external quality assurance programme (EQAP) for the laboratories.

## **5.2 Iron Deficiency Anaemia (IDA) group**

One of the objectives of this study was to measure Hepcidin levels together with other makers of iron deficiency anaemia (IDA) such as Hb, MCV and MCHC in patients with IDA. The measured parameters were then compared to those obtained in the reference normal range group. The other specific objective was to characterise the quantitative behaviour of Hepcidin. This was done by assessing the associations of Hepcidin with the obtained parameters in the pre-iron IDA group and then follow up the same group after iron treatment. In some instances, the effect of various parameters on Hepcidin was also assessed using data which encompassed values from both the healthy and the iron deficient participant groups.

### **5.2.1 Haemoglobin, iron and Hepcidin values in iron deficiency anaemia**

Figure 4.3 indicate that haemoglobin, serum iron and Hepcidin values were consistent with iron deficiency states. The haemoglobin values were below 125g/L which is the threshold for defining anaemia at NamBTS (MoHSS 2012). Full blood count parameters, in particular, haemoglobin levels remain the hallmark for defining anaemia as such these results are important in diagnosis of iron deficiency anaemia. Other parameters such as haemoglobin which are currently in routine use to assess

iron bioavailability could benefit from the introduction of Hepcidin testing because of Hepcidin's response. To illustrate this further, an excerpt from the regression analysis output shown in Table 4.6 indicated that for every 1g/dL increase in haemoglobin, Hepcidin will increase by 5.19ng/mL while holding other parameters constant. Such a magnified increase could help amplify small changes in haemoglobin levels.

From Table 4.7 it can be noted that the entire mean values for serum iron, haemoglobin and Hepcidin are reduced. The median values are also below the lower limits of normal and this is expected in iron deficiency anaemia. The mean hepcidin levels is almost half the lower limit of normal as determined from the reference normal range group. On the other hand, the mean serum iron level is reduced by a slightly higher magnitude comparing it to the lower limit of normal for serum iron in both genders.

The modal value for serum iron was 14.66µg/dL but there was no mode for Hepcidin as all samples yielded unique values. The mean and median values for Hepcidin differ moderately. The mean was larger than the median indicating that the data is positively skewed. The use of logarithmic values of Hepcidin however corrects the skewedness as the mean and median values of log Hepcidin became much closer. It may be useful to introduce the use of logarithmic values of Hepcidin in place of the absolute Hepcidin values because the log values correct for skewedness.

A closer look at the graph in Figure 4.3 shows some relationship between Hepcidin, serum iron and haemoglobin levels. The haemoglobin levels are mostly around 10g/dL (100g/L) and their levels are between those for serum iron and Hepcidin in most cases. There are however instances where the haemoglobin values are higher than serum iron levels. These comparisons were made on values obtained when using conventional units of measurement commonly reported in routine medical diagnostic laboratories. Serum iron levels show the highest peaks with most of them above 20µg/dL but below 60µg/dL. All the serum iron values are below the lower limit of normal for a healthy adult which is 58.4µg/dL. This further confirms that the participants recruited into this study had low iron levels. The results that followed in Tables 4.3, 4.4 and 4.5 gave more information on the associations between the

studied parameters.

From the results presented in chapter 4, it is encouraging to note that Hepcidin emerged as a useful biomarker in this study. Before treatment with iron, the values for Hepcidin and serum iron were low. The Hepcidin levels were much lower relative to serum iron. The results show that Hepcidin levels are reduced in iron deplete states, a finding that is supported by Collins et al., (2008) and Nemeth & Ganz (2009). In this iron deficient state, Haemoglobin showed a better correlation to Hepcidin ( $r=0.26$ ) than serum iron ( $r=0.10$ ) as seen in Table 4.3. It is important to note that these correlations were weak. The weak positive associations could be explainable by the existence of other contributory factors in haemoglobin synthesis. These factors are due to the presence of complex physiological mechanisms available for red blood cell production. Haemoglobin synthesis directly relies on the presence of adequate amounts of iron.

Studies by Takasawa et al., (2015) yielded slightly higher ( $r=0.44$ ) correlation values for haemoglobin and Hepcidin which showed a moderate association between these two parameters. Studies by Naqvi et al., (2016) however also showed weak correlations in iron deficiency anaemia with serum iron and Hepcidin showing a correlation ( $r$ ) of 0.25. In this current study as well as the one highlighted, all the correlations were not strong. This highlights the significant role played by other factors in haemoglobin synthesis and this could attribute to reduced levels of association with the two named parameters. Inadequate iron bioavailability results in the formation of less haemoglobin and this has a knock-on effect on the quality of red cells produced which are usually less haemoglobinised and smaller in size (Hoffbrand and Moss 2011). This morphological feature is usually termed a microcytic hypochromic red cell picture. Furthermore, microcytosis and hypochromasia directly affect red cell indices such as Mean Corpuscular Haemoglobin (MCV) and Mean Corpuscular Haemoglobin Concentration (MCHC).

To confirm the contribution of factors other than iron and Hepcidin in the quantitative behaviour of haemoglobin a multiple regression analysis including MCV and MCHC was made. Multiple regression analysis taking into account red cell indices such as MCV, serum iron and Hepcidin yielded an overall  $r$  value of 0.77 further supporting the multifactorial associations of haemoglobin and other parameters. Multiple regression analysis is useful in predicting the value of a

dependant variable based on the value of at least two multiple independent variables. On the other hand, correlation analysis measures the strengths of associations between two variables. The simple correlations carried out (Table 4.3) in this study managed to place Hepcidin as a better test than serum iron on the basis of the strength of its association with haemoglobin levels.

Generally, the studied association between haemoglobin and Hepcidin ( $r=0.26$ ) shown in Table 4.3 is in line with observed values established elsewhere. With the analysis it was possible to see if the defined variables (Hepcidin and haemoglobin as well as serum iron and haemoglobin) move in conjunction with each other. The logarithmic values of Hepcidin and serum iron were also correlated with haemoglobin. The use of logarithmic values corrects the skewedness that can be brought about by large values and therefore helps to show a clearer picture. Serum iron and Hepcidin values in particular have a wide range with reference normal Hepcidin levels stretching from 17.80ng/mL to 86.18ng/mL. Logarithmic values therefore become useful in clarifying the associations. Furthermore, the use of logarithmic values of Hepcidin resulted in a statistically significant, relatively moderate association with haemoglobin ( $r=0.37$   $p=0.02$ ) in this participant group. The use of Hepcidin logarithmic values therefore seems to offer more diagnostic value as it associates better with haemoglobin levels.

In addition to the effects on haemoglobin, this current study has also investigated the effects of various parameters on Hepcidin. Iron and haemoglobin were found to have relatively high positive associations of  $r=0.710$ ;  $r=0.62$  respectively with Hepcidin when looking at a combined group of iron deficient and non-iron deficient participants. This is shown in Table 4.5 and it confirms that the behaviour of Hepcidin is positively influenced by iron in situations where iron deficiency is not present, as well as where there is not enough iron available for normal physiological functions. The results therefore suggest that it is possible to assess the trend in iron bioavailability using Hepcidin levels.

On the contrary, weak to moderate associations between Hepcidin and serum ferritin were reported by Galesloot et al., (2011). Elgari et al., (2015) also reported similar weak positive correlations in paediatric patients with iron deficiency anaemia. Naqvi et al., (2016) reported similar ( $r=0.25$ ) weak association between Hepcidin

and serum iron. The weaker associations were reported in exclusively iron deficient groups. Compared to a homogenous group assessed in this study, the weaker correlations highlight the diminishing correlation which accompanies anaemic states. However, the consistent associations reported in the iron deficient study populations confirm that Hepcidin has a steady and constant relationship with serum ferritin as an indicator of iron bioavailability. Serum ferritin and serum iron measurements assess iron bioavailability and their relationship with Hepcidin is through a common hepcidin ferroportin axis (Nemeth 2010) making their correlations to Hepcidin comparable. In this regard, the use of serum Hepcidin may in no doubt add value to the diagnosis of iron deficiency anaemia.

In the same analysis shown in Table 4.5 serum iron levels showed stronger correlations ( $r=0.74$ ) than Hepcidin ( $r=0.62$ ) to haemoglobin. This helps to explain the role played by iron bioavailability in erythropoietic activity, in particular haemoglobin synthesis. However, the dynamic regulation of Hepcidin by iron enables Hepcidin to be a highly sensitivity test in detecting IDA as shown in Table 4.9. Dispositioning iron in this association, Hepcidin is a useful indicator of haemoglobin levels which could be relied upon. It is also pertinent to note that much higher associations were also found between haemoglobin and other full blood count parameters as indicated on Table 4.5. These however are part of the full blood count results and should be tested for in a diagnostic work up for all forms of anaemia. The use of Hepcidin testing would additionally be appropriate for differential diagnosis of iron deficiency anaemia.

It is pertinent to note that the state of the severity of anaemia in most of the participants within the study population was not dire. The samples were obtained from a population of participants with mostly mild and moderate anaemia according to the WHO (2011) classification of anaemia. According to the WHO (2011), mild anaemia is defined as haemoglobin levels ranging from 110.0 to 129.0 g/L for men and 110.0 to 119.0 g/L for women. Moderate anaemia is defined as haemoglobin levels between 80.0 and 100.9 g/L for both sexes and severe anaemia, <80.0 g/L for both sexes as well (WHO 2011). It may therefore be necessary to find out how both Hepcidin and serum iron correlate in severe anaemia in future studies. The current selection of participants is however adequate for the purpose of answering the research questions presented.



In future studies, larger sample sizes which include participants with a wider variety of the degrees of anaemia would be more effective in confirming the usefulness of Hepcidin. This could be done as a prospective study where samples are tested for both Hepcidin and current parameters in the actual medical laboratory setting. The results could then be collated over a longer period of time giving much higher sample sizes which are otherwise difficult to attain. The current calculated sample size however managed to yield results which were sufficient to achieve the objectives of this research.

#### **5.2.2. Effects of serum iron, Hepcidin and other factors on haemoglobin:**

An analysis in IDA patients before commencing iron therapy

The correlation analysis on the pre-iron treatment group shows a correlation ( $r$ ) value of 0.10 which shows a very weak positive association between serum iron levels and haemoglobin values. The  $p$  value of 0.54 however indicates that this is not statistically significant at 5% level of significance. On the contrary, in a correlation analysis independent of iron, a weak positive correlation ( $r=0.26$ ) was obtained between Hepcidin and Hb values. Although the  $r$  value for Hepcidin was weak at 0.26 it however appears better than that for serum iron, an analyte that is already in routine use for the diagnosis of this ailment. Furthermore, the log Hepcidin correlation with haemoglobin levels showed a statistically significant association ( $r=0.37$   $p=0.02$ ). These results; highlighted in Table 4.3b above, enables us to underline the superior quality of Hepcidin over serum iron despite serum iron being one of the current routine diagnostic tests for iron deficiency anaemia.

The  $r$  value for log Hepcidin was 0.37 which shows a relatively stronger relationship for logarithmic values of Hepcidin on haemoglobin levels. Together with the  $R^2$  values for iron and Hepcidin shown in Table 4.3 (a & b), the results suggest that the contribution of Hepcidin (6.9%) to haemoglobin values is better than that for serum iron (1%). This finding makes a reasonable contribution to the case supporting the use of Hepcidin in routine diagnosis of anaemia due to iron insufficiency. These findings support the notion that the diagnostic accuracy of Hepcidin in iron deficiency anaemia is better than that of serum iron, again a case supporting the use of Hepcidin in routine diagnosis. The contribution of the logarithmic values of

Hepcidin (13.4%) to haemoglobin is even higher, again supporting the notion echoed above that Hepcidin logarithmic values offer better diagnostic value .

The relatively low correlation values of Hepcidin and iron to haemoglobin in the IDA participant group may possibly be attributed to the fact that the relationship is influenced by other factors such as erythropoietic activity. The nutrient status backing up the erythropoietic activity dictates the ultimate morphological features of red cells. Features such as microcytosis and hypochromia are affected by nutrient iron availability and therefore has an effect on the full blood count indices of the red blood cells so formed. Furthermore, an increase in erythropoietic activity results in increased erythroid mass which consequently produces more erythroferrone, a hormone which suppresses Hepcidin transcription. It therefore becomes important to find out the role played by Hepcidin, serum iron as well as other factors attributable to erythropoietic activity in predicting the presence or absence of iron deficiency anaemia. Some of the other factors attributable to erythropoietic activity are the red cell indices namely MCV, MCHC, RDW<sub>cv</sub>, MCH and HCT.

To find out the role of these multiple dependent variables, a multiple regression analysis was carried out. The outputs from the multiple regression analysis are shown in Table 4.4. the r overall (multiple R) of 77.05% shows that that the combined effect of parameters such as red cell count, serum iron levels, Hepcidin levels and red cell indices have a reasonably high effect on the variability of Haemoglobin. This confirms the explanation on erythropoietic activity given above.

### **5.2.3. Iron treatment in the IDA participant group.**

Iron treatment resulted in an increase in serum iron, Hepcidin and haemoglobin levels. This was as expected and the data in Table 4.8 confirms that there was a positive response to iron therapy. The pre-iron and post-iron treatment differences are significant as indicated by the p-values obtained which are all very close to zero. The haemoglobin levels for all the participants rose by more than 20g/L. an increase of at least 20g/L is the threshold set as a benchmark to define successful response to iron treatment (Alleyne et al 2008). Furthermore, Hoffman et al (2013) suggest that a 20g/L increase in haemoglobin should be evident after three weeks.

The response to iron treatment experienced in this study confirmed an increase in iron bioavailability as indicated by increased serum iron levels. The selected participant group therefore managed to set the right base for studying the behaviour of Hepcidin in iron deficiency anaemia patients undergoing treatment. Non-response to iron treatment could be an indication of the presence of other forms of anaemia other than IDA (Hoffbrand and Moss 2011). This was however not the case and a positive response managed to further qualify the participant group as an IDA participant group.

Hepcidin expression is suppressed to facilitate iron uptake (Pasricha et al., 2014) and this was the case as evidenced by the depressed Hepcidin values before iron therapy. The Hepcidin values returned to normal in the post iron treatment group presumably due to the iron replete state found in this group. Such an observation is reassuring considering the importance of the dynamics of Hepcidin in the prognosis of IDA patients. Hepcidin plays an important role in maintaining a physiological balance for iron (Ganz and Nemeth 2012). Its concentration is in turn regulated by the serum iron levels and this makes it a sensitive indicator of iron bioavailability.

To further elucidate the diagnostic and prognostic value of Hepcidin, its sensitivity, specificity and predictive values were assessed by carrying out receiver operating curve (ROC) analysis. This was performed by looking at the specificity and sensitivity of Hepcidin in the iron deficiency anaemia group before treatment and in the same group after treatment. Hepcidin levels below the lower limit of normal were regarded as abnormal and indicative of iron deficiency. Hepcidin levels within the reference normal range were regarded as normal and were expected in non-iron deficient states such as those after iron treatment. The ranges used were those determined for the Namibian blood donor population in the previous chapter.

The outputs from the ROC analysis such as sensitivity and specificity results were presented in Table 4.9. The results showed a very high specificity and sensitivity of 100% (CI 91.19; 100.00) and 85% (CI 70.16; 94.29) respectively when using absolute Hepcidin levels. The positive predictive value (100% CI 89.72; 100.00) and negative predictive value (86.96% CI 73.74; 95.06) for Hepcidin are also high.

It must be noted however that these are dependent on the prevalence of the disease in a particular population.

In this calculation, the predictive values assume a prevalence rate of 50%. This is an unpretentious indication of the prevalence of iron deficiency anaemia among anaemic patients in Namibia. The average global incidence of iron deficiency anaemia among anaemic patients is 50% (Stoltzfus 2003). Prevalence data for Namibia (WHO 2001; WHO 2011; WHO 2015) estimate the prevalence to be 40-50%. In fact, iron deficiency is so common in anaemic patients that some authors (UNICEF/Namibia 2006 page 18) refer to all forms of anaemia as iron deficiency anaemia. This may however be misleading as other forms of anaemia are also found in the Namibian population.

The discriminative ability of Hepcidin has been confirmed by the measures of diagnostic accuracy indicated in Table 4.9 above. The findings highlight the utility of Hepcidin as a diagnostic test for iron deficiency anaemia. Furthermore, the use of logarithmic values of Hepcidin tends to give better results in correlation analysis as indicated in Table 4.10. It may be an indication that although Hepcidin is proving to be a better marker, its logarithmic values may have a superior utility in diagnosing IDA.

#### **5.2.3.1. Observed trends in the prognosis of Iron Deficiency Anaemia**

Treatment of IDA by replenishing iron stores corrects the condition and this can be monitored using biochemical markers such as Hepcidin and serum iron levels. An increase in serum iron and Hepcidin levels is regarded as an indication of treatment success when monitoring the prognosis of IDA. However, increasing erythropoietic activity suppresses Hepcidin expression (Ashby et al., 2010) while increasing iron levels inhibit the suppression of Hepcidin expression (Ganz et al., 2008). There is an initial increase in Hepcidin levels whenever serum iron increases. This makes Hepcidin a sensitive marker of iron bioavailability. In this study Hepcidin levels increased after iron therapy. Results from this study were consistent with Ganz et al's (2008) notion, our mean Hepcidin levels rose from 9.848ng/mL to 79.851ng/mL after iron treatment. The mean Hepcidin value increased nine times after iron treatment and mean iron levels increased four times from base line iron in IDA.

The magnitude of increase in Hepcidin levels seem to point out a more pronounced response to iron levels. With Hepcidin having an immediate effect on iron levels (Rivera et al., 2005) it is possible that it can be used as a sensitive indicator of iron bioavailability. The constant associations of Hepcidin to haemoglobin pre- and post-iron therapy, albeit low, seems to also support the notion that Hepcidin is sensitive to the dynamics of iron utilisation in haemoglobin formation.

Absence of microcytosis does not exclude IDA because this morphological feature will appear later (Hoffbrand and Moss 2011). On the contrary presence of microcytosis is not always indicative of IDA because some forms of anaemia such as those due to haemoglobinopathies can also present with a microcytic blood picture. In such cases red cell indices, in particular MCV have very limited prognostic value. Hepcidin levels however tend to increase regardless of the red cell indices but in response to levels of iron bioavailability. This feature makes Hepcidin a better marker for iron bioavailability.

Table 4.12 show the correlations between Hepcidin, serum iron,  $\Delta$  Hepcidin,  $\Delta$  serum iron and haemoglobin before and after iron treatment. Serum levels of Hepcidin before iron treatment show a moderate correlation with haemoglobin in both pre ( $r= 0.26$   $p= 0.546$ ) and post ( $r= 0.24$   $p= 0.546$ ) treatment groups. The pre-iron treatment Hepcidin associations did not reach statistical significance as indicated by the  $p$  values in Table 4.12. The serum iron levels before treatment with iron however had lower correlation values with pre-treatment haemoglobin ( $r=0.10$   $p=0.838$ ) and post-treatment haemoglobin ( $r=0.05$   $p=0.383$ ). Serum Hepcidin levels before treatment however showed a moderate positive association ( $r=0.46$   $p=0.546$ ) with pre-treatment serum iron levels. Similarly, the  $\Delta$  serum iron showed a moderate positive association ( $r=0.25$ ) with  $\Delta$  Hepcidin confirming the consistent association of baseline Hepcidin with serum iron.

Another finding worth noting was the moderate negative correlation ( $r= -0.36$ ) between baseline Hepcidin and  $\Delta$  serum iron levels. Also, worth noting is the fact that other factors such as erythropoietic activity have an effect on the change in haemoglobin levels which subsequently have an effect on the behaviour of Hepcidin.

In this study there is a stronger association of Hepcidin with serum iron ( $r= 0.46$ ) in iron deficiency anaemia before treatment compared to iron replete states after treatment as shown in Table 4.11. The Hepcidin serum iron correlation post iron treatment was lower ( $r=0.22$ ) and it appears the correlation coefficients for Hepcidin were halved after treatment. The stronger correlations in iron deplete states has also been pointed out by Elgari et al., (2015). Bregman et al., (2013) also concurs with this finding that Hepcidin correlations with iron are stronger in iron deficiency and attributes this to increased erythropoietic activity amongst other factors. The relatively stronger correlation values in the pre-treatment IDA participant group could also be attributed to the dynamics of iron utilisation in iron deplete states where erythropoietic activity is either stable or slowing down.

Another interesting finding in Table 4.11 is the correlation values for pre-iron treatment Hepcidin levels with haemoglobin values. The pre-iron treatment Hepcidin levels and pre-iron treatment haemoglobin levels had a correlation value ( $r$ ) of 0.26. The same pre-iron treatment Hepcidin levels and post treatment haemoglobin levels had an  $r$  value of 0.24. The association of pre-iron treatment Hepcidin seems to be constant with both pre-treatment and post-treatment haemoglobin levels. Although the associations are weak it is important to note that these remain constant regardless of the iron status.

### **5.3 Hepcidin testing in Iron Deficiency Anaemia (IDA).**

Results obtained in chapter 4 indicate that the quantitative behaviour of Hepcidin is consistent with expected findings in different iron states. In IDA, Hepcidin is reduced and it returns to normal in iron replete or non-IDA states. This confirms that this biomarker can be used as a diagnostic tool in IDA. ROC analysis showed that Hepcidin is both a sensitive and specific test in IDA. It is also encouraging to note that the magnitude of change in Hepcidin is amplified compared to haemoglobin and other full blood count parameters. Small changes in haemoglobin can therefore be detected and are more pronounced when Hepcidin values are used to interpret these changes on patients undergoing treatment for IDA. In all these findings, Hepcidin has shown to be a test worth considering if diagnosis of IDA is to be improved.

## CHAPTER 6

### 6. GENERAL CONCLUSION AND RECOMMENDATIONS

#### 6.1. Discussion

The discovery of Hepcidin has presented new opportunities in the diagnosis and treatment of disorders associated with iron regulation such as iron deficiency anaemia. Since its discovery, a lot of work has gone into understanding the characteristics of Hepcidin as well as its behaviour. Recently Gonzo et al., (2017) established reference normal ranges for Hepcidin in the Namibian blood donor population. This work was the first of its kind in Namibia, a country in the southern part of Africa. However, not much has been done in trying to introduce it into the mainstream diagnostic arena for iron deficiency anaemia. The usefulness of Hepcidin in patients with iron deficiency anaemia has more hope and this study has demonstrated that it is worth considering Hepcidin testing for both the diagnosis and prognosis of iron deficiency anaemia.

This study, to my knowledge was the first to characterise serum Hepcidin concentrations in Namibia more so in the Namibian blood donor population. The study firstly established reference normal ranges for the adult populace in Namibia using the Namibia blood donor population (Gonzo et al., 2017). This work has been published in the International Journal of Blood Transfusion and Immunohematology (IJBTI). The established reference normal ranges are comparable to those established elsewhere (Galesloot et al., 2011; Sdogou et al., 2015) affirming a reasonable level of external validity.

Following on from establishing the reference normal ranges, characterisation of Hepcidin in iron deficiency anaemia (IDA) was carried out. From other studies, Hepcidin levels are depressed in iron deplete states (Collins et al., 2008; Nemeth & Ganz 2009) and this is consistent with findings from this study. The Hepcidin levels were suppressed in the pre-iron treatment IDA study group. Serum iron and

haemoglobin levels were also reduced indicating the presence of an iron deficient state. In this state, haemoglobin levels demonstrated a moderate positive correlation ( $r=0.26$ ) with Hepcidin.

Although the association was moderate, it was stronger than between haemoglobin and serum iron which was  $r=0.10$ . The analysis therefore confirmed the presence of a relationship between Hepcidin and current markers of iron bioavailability.

Hepcidin values in iron deficiency and iron replete states were found to be significantly different in the population under study confirming its diagnostic ability to discriminate between iron deficiency and healthy states. In the post iron treatment IDA group both serum iron and haemoglobin levels were increased confirming a correction of both iron bioavailability and anaemia. It can be inferred that the correction of anaemia was a result of the correction of iron levels. This observation confirms the presence of iron deficiency anaemia in the initial IDA study population as well the effectiveness of the iron treatment intervention in this study population.

The physiological mechanisms involved in Hepcidin regulation are mainly dependent on iron bioavailability (Knutson 2010). This makes Hepcidin levels a more specific index for iron status. Reduced Hepcidin levels are associated with reduced iron bioavailability and increased erythropoietic activity (Ganz et al., 2008). Inversely, a correction of depressed Hepcidin expression indicates improved iron bioavailability as well as decelerated erythropoietic activity and this would occur once IDA is corrected. In this study, an increase in Hepcidin levels was noted in the post iron treatment study population as indicated by an increased mean Hepcidin level in Table 4.8. In this same study, Hepcidin has demonstrated that its association with haemoglobin is comparable to that of the currently used measures of iron bioavailability. This makes it a potential candidate for the diagnosis and prognosis of iron deficiency anaemia.

Increments in haemoglobin and serum iron, (as seen in Figure 4.5 and 4.6) shows that serum iron increased by a higher magnitude compared to haemoglobin levels. On average, the percentage increase in serum iron level was 353.2% compared to 14.6% for haemoglobin levels. These are significant increases, furthermore the



magnitude of iron increments is worrying if the risk of iron overload is put into perspective. In their review, Alleyne et al., (2008) highlighted that haemoglobin levels are expected to increase by about 20g/L within 4 weeks for patients on iron supplementation. To avert the risk of iron overload in patients undertaking iron therapy it may be pertinent to rely on the dynamics of Hepcidin. Hepcidin increments post iron treatments are more pronounced than serum iron levels for the same amount of iron administered. Moreover, Hepcidin correlations with haemoglobin were found to be better compared to iron-haemoglobin associations. We may therefore be able to ascertain adequacy of iron by using Hepcidin testing.

The use of logarithmic values of Hepcidin may even be more appealing because they give better correlations with haemoglobin levels. The improved correlations found with logarithmic values of Hepcidin are due to the fact that data which is presented as logarithms is less skewed. In our case the correction of the degree of skewedness resulted in a more linear relationship and thus higher correlation values. This highlights the superior utility of log Hepcidin compared to absolute Hepcidin values.

Hepcidin levels may also be useful in the differential diagnosis of pure IDA and IDA in anaemia of chronic disease (ACD). In ACD conditions such as irritable bowel syndrome and cancer related anaemia, IDA can arise as a result of gastrointestinal bleeding and the use of anti-cancer treatment regimens (Shu et al., 2015; Bergamaschi et al., 2013). In such cases Hepcidin is upregulated in pure ACD but suppressed in ACD with concomitant iron deficiency (Theurl et al., 2009). With such dynamics in Hepcidin levels, it may be possible to use it to clarify the aetiology of IDA, this has however not been pursued in this study.

In summary, Hepcidin concentrations are related to levels of iron bioavailability and the association exists in both iron deplete as well as iron replete states. Hepcidin levels progressively rise as haemoglobin levels increase indicating a transition from iron deplete to iron replete state. The rise in Hepcidin levels is however more pronounced than in haemoglobin. This is a useful attribute for monitoring response to treatment in patients with IDA.

## **6.2. Conclusion**

The use of Hepcidin in a routine diagnostic setting has great potential. In this study it was possible to establish reference normal ranges as well as demonstrate the quantitative differences of Hepcidin levels in diseased and non-diseased states. The expected Hepcidin levels in both iron replete and iron deficient states were consistent with what was found in this study further confirming its ability to distinguish between IDA and non-IDA states.

This study determined the sensitivity and specificity of Hepcidin at the lower limit of normal as defined by the reference normal range established by the same study. These findings indicated that Hepcidin has very high sensitivity (100%) and specificity (85%) for IDA. Such findings support the notion for introducing Hepcidin into the routine diagnostic arena. Furthermore, the predictive values, both positive and negative, were found to be high. This highlights the fact that Hepcidin testing is able to confirm the presence of IDA in diseased patients as well as ruling out its presence in non-IDA patients.

A potential limitation of Hepcidin as a routine test for the diagnosis of iron deficiency anaemia is the inherent diurnal variation (Nemeth 2010; Kroot et al., 2009). This constraint is common with most hormones (van Kerkhorf et al., 2015) and proper sampling will help to overcome this impediment. Furthermore, once all pre-analytical variables for Hepcidin are established, sample preparation can be tailored to minimise them. This exercise is a common practice in medical diagnostics and will not be unique to Hepcidin testing

## **6.3. Recommendations**

Based on the findings in this study, work towards implementing Hepcidin testing in routine diagnosis for iron deficiency anaemia should continue. Before the full implementation of Hepcidin testing in Namibia the following considerations should be looked at:

### **6.3.1. Hepcidin testing**

Implementation of Hepcidin testing primarily for the diagnosis and prognosis of iron deficiency anaemia along with current testing methods is recommended. The rationale for the implementation is to enable the validation of this test in the new environment and to enable collection of more data on the behaviour of Hepcidin in iron deficiency anaemia in different clinical settings. In implementing routine Hepcidin testing laboratories should consider the use of reference preparations of Hepcidin to quality control their runs. The reference preparations would also enable inter-laboratory comparison of assay runs, a motion which has also been echoed by Pasricha et al., (2011)

### **6.3.2. Reference normal range**

Establish reference normal ranges which are stratified by age and gender to enable interpretation of results in the respective demographic scenarios. This should be prioritised. To fulfil this, a more extensive sampling strategy which takes into consideration the populations from different geographical areas within Namibia should be employed. Sampling should transcend beyond the boundaries of the Namibian blood donor community.

In conclusion the use of Hepcidin testing should be highlighted and encouraged in the Namibian treatment guidelines. The Namibian treatment guidelines (MoHSS 2011) is the medium in which the country communicates guidelines on the diagnosis and management of disease including iron deficiency anaemia.

### **6.3.3. Further testing**

Further testing should be carried out which investigates the correlations between reticulocyte parameters and Hepcidin in the Namibian population. The study will be useful in assessing erythropoietic activity in an IDA setting.

## CHAPTER 7

### 7. BIBLIOGRAPHY

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## APPENDICES

### 8. APPENDICES

#### Appendix 1: Serum iron kits insert showing the protocol for serum iron analysis



**Iron**  
FerroZine. Colorimetric  
Quantitative determination of iron

##### PACKAGING

Ref.: 101-0374	Cont.: 4 x 50 mL
Ref.: 101-0455	Cont.: 12 x 50 mL
Ref.: 101-0625	Cont.: 4 x 250 mL

Store at 2-8° C

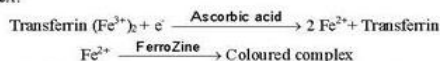
##### CLINICAL SIGNIFICANCE

The iron is the component of a great number of enzymes. The myoglobin, muscular protein, contains iron, as well as the liver. Iron is necessary for the hemoglobin production, molecule that transports oxygen inside red globules. Their deficit in the last causes the ferropenic anemia. High levels of iron are found in hemochromatosis, cirrhosis, hepatitis and in increased transferrin levels. The variation day to day is quite marked in healthy people<sup>1,5,6</sup>.

Clinical diagnosis should not be made on a single test result; it should integrate clinical and other laboratory data.

##### PRINCIPLE OF THE METHOD

The iron is dissociated from transferring-iron complex in weakly acid medium. Liberated iron is reduced into the bivalent form by means of ascorbic acid. Ferrous ions give with FerroZine a coloured complex:



The intensity of the color formed is proportional to the iron concentration in the sample<sup>1,2</sup>.

##### REAGENTS

R 1	Buffer	Acetate pH 4.9	100 mmol/L
R 2	Reductant	Ascorbic acid	99.7 %
R 3	Color	FerroZine	40 mmol/L
IRON CAL		Iron aqueous primary standard	100 µg/dL

##### Optional (not included in the kit)

Contro-N	Ref.: 101-0252	4 x 5 mL	Lyophilized human control serum
	Ref.: 101-0083	20 x 5 mL	
Contro-P	Ref.: 101-0253	4 x 5 mL	Lyophilized human control serum
	Ref.: 101-0084	20 x 5 mL	

##### PREPARATION

Working reagent (WR):

Dissolve (→) the contents of one tube R 2 Reductant in one bottle of R 1 Buffer.

Cap and mix gently to dissolve contents.

Stability: 3 months at 2 - 8° C or 1 month at 15 - 25° C.

##### STORAGE AND STABILITY

All the components of the kit are stable until the expiration date on the label when stored tightly closed at 2 - 8° C, protected from light and contaminations prevented during their use.

Do not use reagents over the expiration date.

##### Signs of reagent deterioration:

- Presence of particles and turbidity.
- Blank absorbance (A) at 562 nm ≥ 0.020.

##### ADDITIONAL EQUIPMENT

- Spectrophotometer or colorimeter measuring at 562 nm.
- Matched cuvettes 1.0 cm light path.
- General laboratory equipment<sup>(Note 1)</sup>.

##### SAMPLES

Serum or heparinized plasma.

Free of hemolysis and separated from cells as rapidly as possible.

Stability of the sample: 2 - 8° C for 7 days<sup>1</sup>.

##### PROCEDURE

Notes: CHRONOLAB SYSTEMS has instruction sheets for several automatic analyzers. Instructions for many of them are available on request.

IRON CAL: Proceed carefully with this product because due its nature it can get contaminated easily.

It is recommended to use disposable material. If glassware is used the material should be soaking for 6 h in diluted HCl (20 % v/v) and then thoroughly rinsed with distilled water and dried before use.

Calibration with the aqueous standard may cause a systematic error in automatic procedures. In these cases, it is recommended to use a serum Calibrator.

Use clean disposable pipette tips for its dispensation.

The reference values are strongly method dependent.

- Assay conditions:  
Wavelength: ..... 562 nm (530-590)  
Cuvette: ..... 1 cm light path  
Temperature: ..... 37° C / 15 - 25° C
- Adjust the instrument to zero with distilled water.
- Pipette into a cuvette:

	WR Blank	Standard	Sample Blank	Sample
WR (mL)	1.0	1.0	1.0	1.0
R 3 (drops)	1	1	--	1
Distilled water (µL)	200	--	--	--
Standard <sup>(Note 2,3)</sup> (µL)	--	200	--	--
Sample (µL)	--	--	200	200

- Mix and incubate 5 min at 37° C or 10 min at room temperature.
- Measure the absorbance (A) of Standard and sample against WR Blank. The colour is stable for at least 30 minutes.

##### CALCULATIONS

(A)Sample - (A) Sample Blank x 100 (Standard conc.) = µg/dL iron  
(A)Standard

Conversion factor: µg/dL x 0.179 = µmol/L.

##### QUALITY CONTROL

Control sera are recommended to monitor the performance of assay procedures.

If control values are found outside the defined range, check the instrument, reagents and calibrator for problems.

Each laboratory should establish its own Quality Control scheme and corrective actions if controls do not meet the acceptable tolerances.

##### REFERENCE VALUES<sup>5</sup>

Male 65 - 175 µg/dL ≅ 11.6 - 31.3 µmol/L<sup>(Note 4)</sup>  
Female 40 - 150 µg/dL ≅ 7.16 - 26.85 µmol/L<sup>(Note 4)</sup>

These values are for orientation purpose; each laboratory should establish its own reference range.

PICC041e  
V 2014/2

CHRONOLAB SYSTEMS, S.L., C/Diputació 260, 08007 Barcelona, SPAIN  
Tel. +34 617722466, www.chronolab.com, e-mail: comercial@chronolab.es

#### PERFORMANCE CHARACTERISTICS

**Measuring range:** From *detection limit* of 1.85 µg/dL to *linearity limit* of 1000 µg/dL.

If the results obtained were greater than linearity limit, dilute the sample 1/2 with NaCl 9 g/L and multiply the result by 2.

#### Precision:

	Intra-assay (n=20)		Inter-assay (n=20)	
Mean (µg/dL)	102	190	107	193
SD	0.88	1.31	1.26	1.57
CV (%)	0.86	0.69	1.18	0.81

**Sensitivity:** 1 µg/dL = 0.0009 A.

**Accuracy:** Results obtained using CHRONOLAB reagents did not show systematic differences when compared with other commercial reagents.

The results obtained using 50 samples were the following:

Correlation coefficient (r): 0.987.

Regression equation:  $y = 1.0052x - 2.3159$ .

The results of the performance characteristics depend on the analyzer used.

#### INTERFERENCES

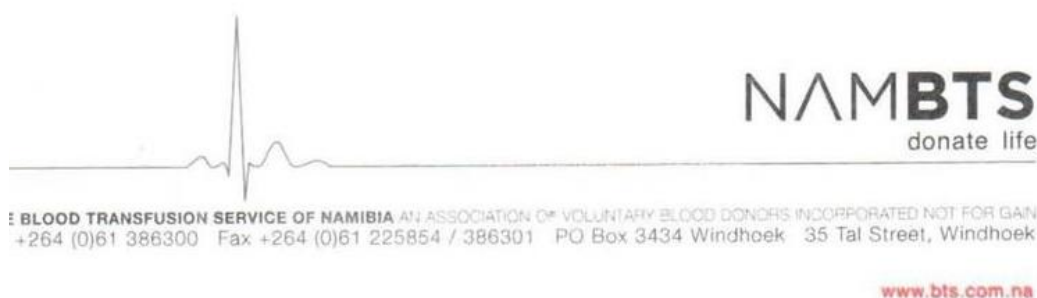
Hemolyzed samples are rejected, since erythrocytes contain iron and therefore falsely elevate the serum results<sup>1,2</sup>.

A list of drugs and other interfering substances with iron determination has been reported by Young et. al<sup>3,4</sup>.

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## Appendix 2: Permission and Ethical clearance letter for NaMBTS



23 October 2015

Mrs Cornelia de Waal-Miller  
Programme Coordinator  
Department of Biomedical Sciences  
School of Health and Applied Sciences  
Polytechnic of Namibia

Cc. Mr Martin Gonzo

Dear Madam

**REF: Request for permission and ethical clearance to use donors specimens from NAMBTS for a research study titled "Usefulness of Hepcidin as a Biomarker in patients with iron deficiency anaemia".**

**The above subject has reference:**

Permission is hereby granted to Mr Martin Gonzo and colleagues to use specimens from blood donors in carrying out the above research. You will be required to provide collection materials e.g needles etc for collecting specimens from the donors who consent to participate in the study and NAMBTS through the Donor Division will collect the specimens and forward to you. Please abide by the following conditions:

1. No publications will be made without permission from NAMBTS.
2. Donor specimens will be used for this study only.
3. NAMBTS will get a copy of the results of the study.

Hoping this is in order.

Kind regards,

Mr. Israel Chipare  
Head of Technical Division  
Blood Transfusion Service of Namibia

**Appendix 3: Permission and Ethical clearance letter from Ministry of Health and Social Services, Namibia.**



**REPUBLIC OF NAMIBIA**

*Ministry of Health and Social Services*

Private Bag 13198  
Windhoek  
Namibia

Ministerial Building  
Harvey Street  
Windhoek

Tel: 061 – 203 2125  
Fax: 061 – 222558  
E-mail: [msimasiku@mhss.gov.na](mailto:msimasiku@mhss.gov.na)

**OFFICE OF THE PERMANENT SECRETARY**

**Ref:** 17/3/3

**Enquiries:** Mr. M. Simasiku

**Date:** 28 July 2016

**Mr. Martin Gonzo**  
Namibia University of Science and Technology  
13 Storch Street  
Private Bag 13388  
Windhoek  
Namibia

Dear Mr. Gonzo

**Re: The Potential usefulness of Hecpidin as a Biomarker in Patients presenting with Iron Deficiency Anaemia(IDA).**

1. Reference is made to your application to conduct the above-mentioned study.
2. The proposal has been evaluated and found to have merit.
3. **Kindly be informed that permission to conduct the study has been granted under the following conditions:**
  - 3.1 The data to be collected must only be used for academic purpose;
  - 3.2 No other data should be collected other than the data stated in the proposal;
  - 3.3 Stipulated ethical considerations in the protocol related to the protection of Human Subjects should be observed and adhered to, any violation thereof will lead to termination of the study at any stage;



- 3.4 A quarterly report to be submitted to the Ministry's Research Unit;
- 3.5 Preliminary findings to be submitted upon completion of the study;
- 3.6 Final report to be submitted upon completion of the study;
- 3.7 Separate permission should be sought from the Ministry for the publication of the findings.

Yours sincerely, \



**Andreas Mwoombola (Dr)**  
**Permanent Secretary**

*"Health for All"*

### Participant Information Sheet for the Hepcidin study

#### Hepcidin Participant Information Sheet



**Title:** The potential usefulness of Hepcidin as a Biomarker in patients with iron deficiency anaemia.

**Dear Participant.**

We are inviting you to take part in a research which aims to find out how best Hepcidin assays can be used to improve the diagnostic and prognostic value of laboratory tests. Hepcidin test is a new test which can be useful in understanding diseases associated with lack of Iron.

Hepcidin and Iron utilisation

Hepcidin is a small molecule that is involved in the way Iron is regulated and made available for blood formation in the body. It plays an important part in the release of Iron and its characteristics can be useful in understanding diseases such as iron deficiency anaemia. Iron deficiency anaemia is a condition whereby the body lacks enough blood cells known as red blood cells.

The red blood cells need iron and other components for their formation. When there is inadequate iron, insufficient amounts of red blood cells are formed and this leads to a condition known as iron deficiency anaemia. When this happens patients are normally given iron to treat the condition.

The introduction of a Hepcidin test for routine use would make it easier to monitor the effectiveness of the treatment. Your participation in this research is very important and we would like to invite you to read and consent to us using your samples for this research.

We can assure you that this is a confidential process and all your samples will be anonyms. This is a voluntary exercise and you are free to withdraw at any time.

Thank you for taking time to read this letter. Please do not hesitate to contact me if you have any queries regarding this research.

Yours faithful

Martin Gonzo (DHealth student)

Supervisors:

Dr Gordon Taylor [mpsgit@bath.ac.uk](mailto:mpsgit@bath.ac.uk)

Dr Aaron Maramba [Aaronm@yahoo.co.uk](mailto:Aaronm@yahoo.co.uk)

Hepcidin Participant Information Sheet\_Martingonzo\_research study

**Deelnemer Inligtingstuk vir die Hepcidin studie**



**Hepcidin Deelnemer Inligtingstuk**

**Titel:** Die potensiele bruikbaarheid van Hepcidin as 'n biomerker in pasiënte met ystertekort anemie

**Geagte Deelnemer.**

Ons nooi u uit om deel te neem aan navorsing wat ten doel het om uit te vind hoe Hepcidin-toetse ten beste gebruik kan word om die diagnostiese en prognostiese waarde van laboratoriumtoetse te verbeter. Hepcidin is 'n nuwe toets wat bruikbaar kan wees om siektes soos ystertekort te verstaan.

Hepcidin en ysterverbruik

Hepcidin is 'n klein molekule wat betrokke is in die manier waarop yster gereguleer word en beskikbaar gestel word vir bloedvorming in die liggaam. Dit speel 'n belangrike rol in die vrystelling van yster en die eienskappe daarvan kan nuttig wees in die begrip van siektes soos ystertekort anemie. Ystertekort anemie is 'n toestand waar die liggaam 'n tekort het aan bloedselle bekend as rooibloedselle.

Die rooibloedselle het yster en ander komponente nodig vir hul vorming. Wanneer daar onvoldoende yster is, word onvoldoende hoeveelhede rooibloedselle gevorm en dit lei tot 'n toestand bekend as ystertekort anemie. Wanneer dit gebeur word pasiënte normaalweg yster toegedien om die toestand te behandel.

Die instelling van 'n Hepcidin-toets vir roetinegebruik sal dit makliker maak om die effektiwiteit van die behandeling te monitor. U deelname in hierdie navorsing is baie belangrik en ons wil u graag uitnooi om te lees en in te stem dat ons u monsters gebruik vir hierdie navorsing.

Ons kan u verseker dat hierdie 'n vertroulike proses is en dat al u monsters anoniem sal wees. Dit is 'n vrywillige oefening en u is vry om te enige tyd te onttrek. Dankie dat u die tyd spandeer het om hierdie brief te lees. Moet asseblief nie huiwer om my te kontak indien u enige navrae oor hierdie navorsing het nie.

Opreg die uwe

Martin Gonzo [mgonzo@nust.na](mailto:mgonzo@nust.na)


Toesighouers:

Dr Gordon Taylor [mpsgit@bath.ac.uk](mailto:mpsgit@bath.ac.uk)

Dr Aaron Maramba [Aaronm@yahoo.co.uk](mailto:Aaronm@yahoo.co.uk)

Hepcidin Participant Information Sheet\_Martingonzo\_research study

## Appendix 6: Consent form for the Hepcidin study-English version

CONSENT FORM-HEPCIDIN STUDY		
Participant Identification Number for this study: .....		
<b>CONSENT FORM</b>		
<b>Title of Project:</b> The potential usefulness of Hepcidin as a biomarker in patients presenting with iron deficiency anaemia		
Name of Researcher: Martin Gonzo		
		Please initial box
1. I confirm that I have read the information sheet dated..... (version.1.2..) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.		<input type="checkbox"/>
2. I understand that my participation will involve blood being taken from me and it is voluntary. I am free to withdraw from the study at any time without giving any reason and my medical care or legal rights will not be affected in any way.		<input type="checkbox"/>
3. I understand that pseudo-anonymised information relating to my medical condition on anaemia and data collected during the study may be looked at by individuals from University of Bath or from regulatory authorities, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records only for the purpose of this research.		<input type="checkbox"/>
4. I understand that the information collected about me will be used to support other research in the future, and may be shared anonymously with other researchers.		<input type="checkbox"/>
5. I agree to take part in the above study and I have been made aware that information collected will be confidentially kept and only used for the purpose of this research.		<input type="checkbox"/>
_____	_____	_____
Name of Participant	Date	Signature
_____	_____	_____
Name of Person taking consent	Date	Signature
Hepcidin Consent form_Martingonzo_research study v1.2		



## Appendix 7: Consent form for the Hepcidin study-Afrikaans version

### TOESTEMMINGSVORM-HEPCIDIN STUDIE



Deelnemer identifikasienommer vir hierdie studie: .....

#### TOESTEMMINGSVORM

**Titel van Projek:** Die potensiele bruikbaarheid van Hepcidin as 'n biomarker in pasiënte wat met ystertekort anemie presenteer

Naam van Navorser: Martin Gonzo

Inisieël asseblief die blok

1. Ek bevestig dat ek die inligtingstuk gedateer ..... (weergawe 1.2.) vir bogenoemde studie gelees het. Ek het die geleentheid gehad om die inligting te oorweeg en vrae te vra en het bevredigende antwoorde hierop gekry. ☐
2. Ek verstaan dat my deelname sal beteken dat bloed van my geneem word, en dit is vrywillig. Ek is vry om te enige tyd van die studie te onttrek sonder om enige rede te verskaf en my mediese sorg en wetlike regte sal nie op enige manier beïnvloed word nie. ☐
3. Ek verstaan dat pseudo-anonieme inligting rakende my mediese toestand van anemie en data wat deur die studie versamel is, moontlik deur individue van die Universiteit van Bath of van beheerliggame ondersoek sal word, waar dit relevant is vir my deelname aan hierdie navorsing. Ek verleen toestemming vir hierdie individue om toegang tot my rekords te hê alleenlik vir die doel van hierdie navorsing. ☐
4. Ek verstaan dat die inligting wat oor my versamel sal word in die toekoms gebruik sal word om ander navorsing te ondersteun, en dat dit anoniem met ander navorsers gedeel kan word. ☐
5. Ek stem in om aan bogenoemde studie deel te neem en ek is bewus gemaak daarvan dat inligting wat versamel word vertroulik gehou sal word en slegs gebruik sal word vir die doel van hierdie navorsing. ☐

Naam van Deelnemer

Datum

Handtekening

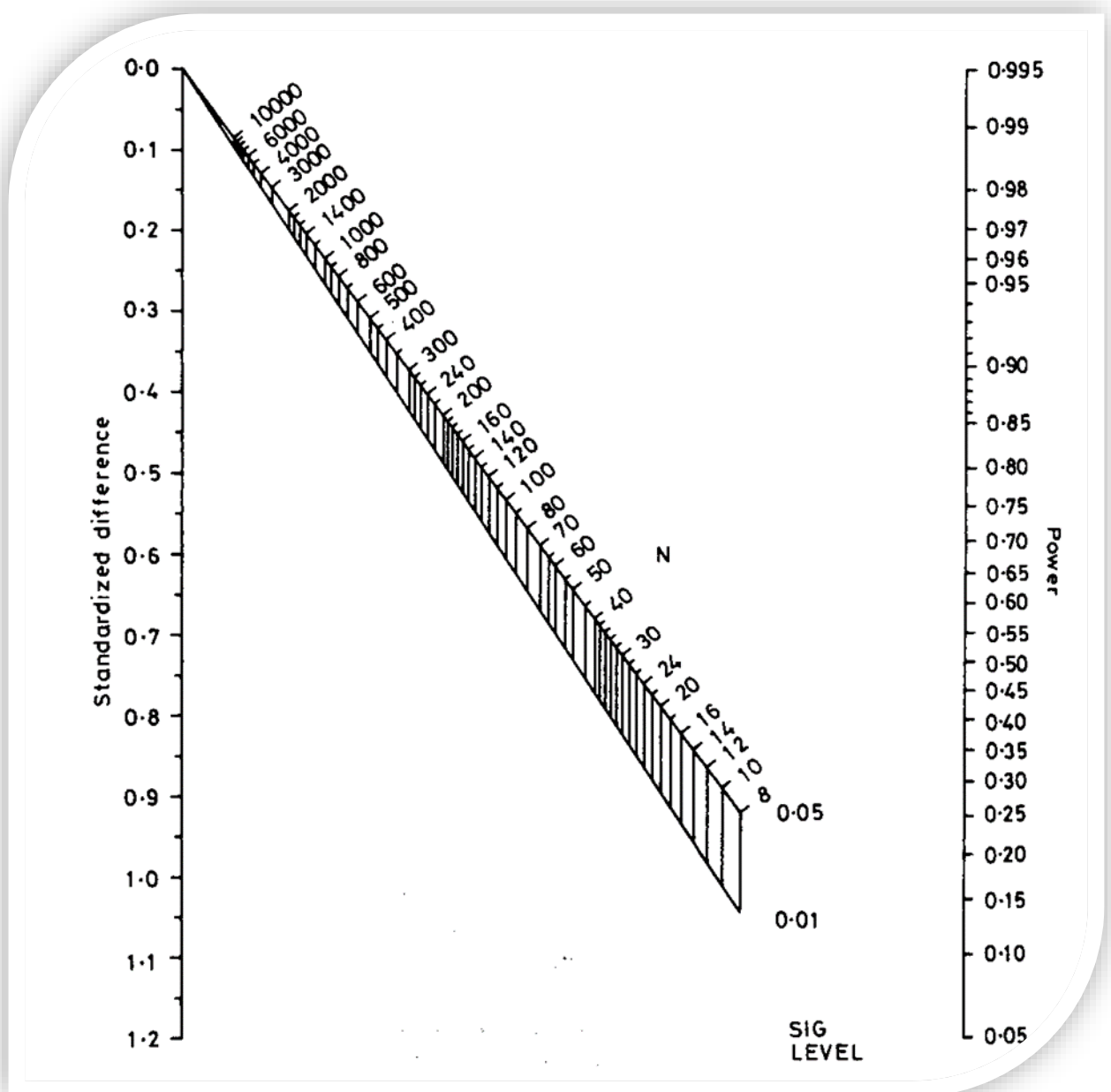
Naam van Persoon wat  
toestemming afneem

Datum

Handtekening

Hepcidin Consent form \_Martingonzo\_ research study v1.2

**Appendix 8:** Altman's nomogram for power and sample size calculation.



Eds S. M. Gore and D. G. Altman. BMA London. Copyright BMJ Publishing Group

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## **PUBLICATIONS**

### **10. PUBLICATIONS**